



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> C07H 21/04, C12Q 1/68 C07K 15/26, C12N 15/12, 15/63 G01N 33/53	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/22331</b>  <b>(43) International Publication Date:</b> 11 November 1993 (11.11.93)
<b>(21) International Application Number:</b> PCT/US93/03936 <b>(22) International Filing Date:</b> 27 April 1993 (27.04.93)  <b>(30) Priority data:</b> 07/874,847 28 April 1992 (28.04.92) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 07/874,847 (CON) Filed on 28 April 1992 (28.04.92)  <b>(71) Applicant (for all designated States except US):</b> THE REGENTS OF THE UNIVERSITY OF MICHIGAN [US/US]; 475 E. Jefferson, Room 2354, Ann Arbor, MI 48109 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> ASTROM, Anders [SE/US]; 3070 Whisperwood Dr., Apartment 428, Ann Arbor, MI 48105 (US). VOORHEES, John, J. [US/US]; 3965 Waldenwood Dr., Ann Arbor, MI 48105 (US). PETTERSON, Ulrika [SE/US]; 3070 Whisperwood Dr., Apartment 428, Ann Arbor, MI 48105 (US). TAVAKKOL, Amir [IR/US]; 1845 Wiltshire, Ann Arbor, MI 48103 (US).  <b>(74) Agents:</b> LEWAK, Anna, M. et al.; Harness, Dickey & Pierce, P.O. Box 828, Bloomfield Hills, MI 48303 (US).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>
<b>(54) Title:</b> HUMAN CRABP-I AND CRABP-II  <b>(57) Abstract</b>  <p>The sequences encoding two isoforms of human cellular retinoic acid binding proteins, CRABP-I and CRABP-II, have been cloned and sequenced and are set forth with their corresponding amino acid sequences in SEQ ID NOS. 1-4. The identification of human CRABP nucleic and amino acid sequences provides the basis for the construction of recombinant human CRABP vectors and expression constructs. Human CRABP can also be synthesized or produced <i>ex vivo</i>, e.g. in bacterial or other production systems. Ligand binding assays, including recombinant and chimeric receptor reporter assays, and direct and competition hybridization assays employing the human CRABP sequences herein described can be used to test drugs for retinoic induction and tissue specificity for pathologies in which retinoids are implicated. Immunoassays utilizing antibodies or binding fragments produced to human CRABP can also be used to test patient tissues for the presence and levels of CRABP for diagnosis and to monitor treatment. The identification of the nucleic and amino acids sequences for human CRABP-I and CRABP-II also contributes to the elucidation of the function and interaction of the retinoid-binding proteins. The CRABP-II gene, isolated from a human placenta genomic library, spans 6 kilobases and includes 4 exons. One major transcription initiation site was mapped to an A residue 137 nucleotides upstream of the ATG initiation codon. CRABP-II mRNA was rapidly induced within 2-6 hours in culture by retinoic acid, primarily due to an increased rate of transcription which required on-going synthesis. The human CRABP-II gene is thus apparently transcriptionally regulated by a newly synthesized regulator protein. Once the CRABP-II is produced, message stabilization may provide means by which elevated CRABP-II in mRNA is maintained.</p>		

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**HUMAN CRABP-I AND CRABP-II****FIELD OF THE INVENTION**

The present invention relates generally to cellular retinoic acid binding proteins (CRABPs) and, more specifically, to human CRABP-I and CRABP-II and the sequences encoding them, and their use in various assay systems for screening and diagnostic applications and for therapeutic purposes.

**RELATED APPLICATIONS**

This is a continuation of U.S. Application Serial No. 07/874,847, entitled "Human CRABP-I and CRABP-II," filed April 28, 1992 by Voorhees et al. which is a continuation-in-part of co-pending U.S. application Serial No. 751,893 entitled "Human CRABP-I and CRABP-II", filed August 30, 1991 by Voorhees et al., all herein incorporated by reference.

**GENBANK ACCESSION INFORMATION**

<u>GENE</u>	<u>ACCESSION NO.</u>
human CRABP-II	M68867

**BACKGROUND OF THE INVENTION**

Retinoids are essential regulators of epithelial cell growth and cellular differentiation, skin being a major target in both normal and pathological states. Sporn, M.B. et al., *Cancer Res.* 43:3034-3040 (1983); Kopan, R. et al., *J. Cell Biol.* 109:295-307 (1989); Asselineau, D. et al., *Dev. Biol.* 133:322-335 (1989); and Lippman, S.M. et al., *Pharmacol. Ther.* 40:107-122 (1989). It has been shown that retinoids prevent cancer in skin and have efficacy as agents in human malignant and premalignant cutaneous disorders. Asselineau, D. et al., *Dev. Biol.* 133:322-335 (1989). It has also been shown that retinoids cause growth inhibition in many hyper-proliferating cell lines, a feature that makes the compounds of fundamental interest as anti-tumor and anti-psoriatic agents. Sporn, M.B. et al., *Cancer Res.* 43:3034-3040 (1983); and Asselineau, D. et al., *Dev. Biol.* 133:322-335 (1989). Retinoids also play fundamental roles in directing the spatial organization of cells during development and the generation of vertebrate limbs. Eichele, G. *Trends Genet.* 5:246-251 (1989); and Summerbell, D. et al., *Trends Neurosci.* 13:142-147 (1990).

The elucidation of the function of retinoids in the complex biological processes involved in cell growth and differentiation requires the identification of the specific components of the retinoid signal transduction system as well as the genes directly regulated by this system. Several intracellular retinoid-binding proteins have already been identified, including cellular retinol-binding proteins (CRBP), nuclear retinoic acid receptors (RAR), cellular retinoic acid binding proteins (CRABP) and, most recently, RXRs, also belonging to the nuclear receptor superfamily of genes. See Sundelin, J. et al., *J. Biol. Chem.* 260:6488-6493 (1985); Li, E. et al., *PNAS (USA)* 83:5779-5783 (1986); Nilsson, M.H.L. et al., *Eur. J. Biochem.* 173:45-51 (1988); Stoner, C.M. et al., *Cancer Res.*

49:1497-1504 (1989); Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990); Petkovich, M. et al., *Nature* 330:444-450 (1987); Brand, N. et al., *Nature* 332:850-853 (1988); Benbrook, D. et al., *Nature* 333:669-672 (1988); Zelent, A. et al., *Nature* 339:714-717 (1989); Krust, A. et al., *PNAS (USA)* 86:5310-5314 (1989); and Mangelsdorf, D.J. et al., *Nature* 345:224-229  
5 (1990).

Cellular retinoic acid binding proteins (CRABP) are low molecular weight proteins present in human skin with increased levels found in psoriatic lesions and after external and systemic retinoid treatment. Siegenthaler, G. et al., *J. Invest. Dermatol.* 86:42-45 (1986); Hirschel-Scholz, S. et al., *Eur. J. Clin. Invest.* 19:220-227 (1989); and Siegenthaler,  
10 G. et al., *Arch. Dermatol.* 123:1690-1692 (1987). Although undetectable in keratinocytes grown in low calcium medium, CRABP is expressed when a more differentiated phenotype is induced by growth to confluence in the presence of elevated extracellular calcium concentrations. Siegenthaler, G. et al., *Exp. Cell Res.* 178:114-126 (1988).

Although the precise role of CRABP in retinoic acid (RA) action has not been  
15 determined, it has been suggested that CRABP might act as a shuttle protein, facilitating the movement of RA to the nucleus, or that CRABP might sequester RA, thereby decreasing the cellular response. Takase, S. et al., *Arch. Biochem. Biophys.* 247:328-334 (1986); Maden, M. et al., *Nature* 335:733-735 (1988). In a recent study, it was found that all biologically active RA analogs in F9 cells bound to RARs, while two of them did not bind  
20 to CRABP. Benbrook, D. et al., *Nature* 333:669-672 (1988). This suggests that retinoid binding to RA, but not necessarily to CRABP is necessary to induce cell differentiation.

The effects of retinoic acid on gene transcription can be mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Leed, M. et al., *Cell* 68:377-395 (1992); Mangelsdorf, D.J. et al., *Genes Dev.* 6:329-344 (1992). RARs have been shown to bind  
25 retinoic acid (RA) with high affinity, while RXRs apparently have no affinity for this ligand. Mangelsdorf, D.J. et al., *Nature* 345:224-229 (1990). However, it was recently demonstrated that 9-*cis* RA can bind to RXR -  $\alpha$  with high affinity. Levin, A. A. et al., *Nature* 355: 359-361 (1992); Heyman, R.A. et al., *Cell* 68:397-406 (1992). CRABPs have been shown to bind RA with high affinity, but their function is poorly understood. However, it  
30 was recently demonstrated that CRABP may be involved in cytochrome P-450 metabolism of RA. Fiorella, P.D. et al., *J. Biol. Chem.* 266:16572-16579 (1991).

Two isoforms of CRABP, CRABP-I and II, have been identified and cloned in the mouse. Stoner, C.M. et al., *Cancer Res.* 49:1497-1504 (1989); Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990); Nilsson, M.H.L. et al., *Eur. J. Biochem.* 173:45-51 (1988). By  
35 isoform is meant two amino acid sequences with substantial sequence identity. Bovine

CRABP-I has also been sequenced, and the NH<sub>2</sub> terminal regions of rat and chicken CRABP-I have also been recently determined. Bailey, J.S. et al., *J. Biol. Chem.* 263:9326-9332 (1988); Kitamoto, J. et al., *Biochem. Biophys. Res. Comm.* 157:1302-1308 (1988). Human CRABP has, however, not been previously isolated or cloned.

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### SUMMARY OF THE INVENTION

The sequences encoding two isoforms of human cellular retinoic acid binding proteins, designated CRABP-I and CRABP-II, and the gene for CRABP-II, have been cloned and sequenced. Their nucleic acid and corresponding amino acid sequences are set forth in the sequence listing preceding the claims. Expression of human CRABP-II, but not  
10 CRABP-I was markedly increased in human skin *in vivo* and in skin fibroblasts *in vitro* after treatment with retinoic acid (RA) and after treatments which induce keratinocyte differentiation. The importance of RA dependent mRNA stabilization for keying CRABP II message at induced levels once transcription has occurred is also described.

The cloning and sequencing of human CRABP provides the basis for the  
15 construction of human CRABP-I and II viral, prokaryotic and eukaryotic expression vectors and recombinant expression constructs. Human CRABP can now also be produced synthetically or *ex vivo* (outside the human body), for example, through the production of fusion proteins in bacteria and later cleavage and purification of CRABP therefrom.

Ligand binding studies utilizing human CRABP sequences can determine ligand  
20 binding affinity and the interaction of human CRABP with other human retinoid-binding proteins, and can be used to better identify tissue-specific drugs for pathologies in which retinoids are implicated. Various assay schemes, including reporter assay systems, direct and competition hybridization and binding assays employ the nucleic and amino acid sequences herein described. Antibodies or binding fragments thereof produced to human  
25 CRABP can also be used in immunoassays of patient tissues for CRABP levels for diagnosis and the monitoring of treatment. Purified or synthetic human CRABP can also be used for supplementation therapy.

Other features and advantages of the present invention will become apparent from the following description and appended claims, taken in conjunction with the  
30 accompanying drawings.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 gives the sequences of the forward and reverse degenerate primers used in cloning human CRABP.

Figure 2 compares human, mouse, rat and chicken CRABP. Panel A is a  
35 comparison of the amino acid sequences of human (h) and mouse (m) CRABP. Dashes

represent sequence identity; the asterisk at residue 118 represents a gap introduced in the CRABP-I sequence for maximum alignment. Panel B is a sequence comparison of the NH<sub>2</sub>-terminal ends of human (h), mouse (m), rat (r) and chicken (c) CRABP. Boxed residues represent those dissimilar to human CRABP-II.

5           Figure 3 is an autoradiogram of RNA blots derived from three individuals which illustrates induction of CRABP-II mRNA in human skin by topically applied retinoic acid (RA). Lane (a) represents no treatments; lane (b) RA vehicle; and lane (c) 0.1% RA cream in RA vehicle under occlusion.

10           Figure 4 is a bar graph of the RNA blot hybridization results (quantitated by laser densitometry) of nine independent experiments involving five dermal fibroblast lines prepared from three individuals and three diploid human lung fibroblast lines. The results illustrate the induction of human CRABP-II mRNA in human dermal fibroblasts compared to lung fibroblasts. The inset shows the relevant autoradiographic bands from two representative experiments comparing dermal and lung fibroblasts.

15           Figure 5 illustrates the expression of CRABP-II mRNA in cultured keratinocytes under various conditions. Figure 5A is a bar graph of the results obtained for seven independent keratinocyte strains and illustrates the effects of confluence on human CRABP-II mRNA levels. The error bars represent  $\pm$  SE \*\*p<0.005, \*\*\*p<0.0005 relative to cells 2 days preconfluence. Figure 5B shows the effects of RA and increased calcium  
20           concentration on human CRABP-II mRNA levels in postconfluent keratinocytes, with treatment for prolonged periods with low concentrations of RA ( $3 \times 10^{-9}$ M) having no detectable effect. Figure 5C illustrates that prolonged treatment of postconfluent keratinocytes with higher concentrations of RA ( $3 \times 10^{-6}$ M) reduced CRABP-II mRNA to undetectable levels.

25           Figure 6 is a schematic diagram of a retinoic acid receptor (RAR)-CRABP or RXR-CRABP cotransfection assay.

          Figure 7 is a restriction map of a bacteriophage lambda clone ( $\lambda$ 2.1) isolated from a human placenta genomic library of the human CRABP-II gene is provided with the exons indicated as filled boxes numbered I to IV.

30           Figure 8 illustrates the nucleotide sequence and shown above the nucleotide sequence is the deduced amino acid sequence at the human CRABP-II gene.

          Figure 9 illustrates the transcription start sites in the human CRABP-II gene as determined by primer extension analysis, with the major transcription site indicated by an arrow and the corresponding base in the sequence indicated by an asterisk.



Figure 10 illustrates a nuclear run-off assay using nuclei isolated from cultured human skin fibroblasts treated with retinoic acid.

Figure 11 illustrates the mRNA level and transcription rate of CRABP-II after quantitation by phosphorimaging and normalization to cyclophilin.

5 Figure 12 is an autoradiogram demonstrating the effects of cycloheximide or actinomycin D on the induction of CRABP-II mRNA expression.

Figure 13 is a bar graph demonstrating the effects of cycloheximide on CRABP-II transcription rates determined by nuclear run-on assays.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

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### OVERVIEW

The cloning and sequencing of two human CRABP cDNAs revealed one with a predicted amino acid sequence 99.3% identical to the mouse and bovine CRABP-I, and a second with a predicted amino acid sequence, 93.5% identical to the mouse CRABP-II. The CRABP-II described herein appears to be the human homolog to mouse CRABP-II, since both are expressed in adult skin, and is therefor designated as such. The high amino acid homology seen between bovine, mouse and human CRABP-I indicates that this isoform has been more conserved throughout evolution than the CRABP-II (Figure 2A described below). This was especially evident when the NH<sub>2</sub>-terminal sequence between rat, chicken, mouse and human CRABP-I and CRABP-II were compared. No differences were seen between the CRABP-I, while several differences exist between the CRABP-IIs.

20 CRABP-I transcripts were undetectable in adult human epidermis by RNA blot hybridization, while the CRABP-II cDNA probe detected an approximately 1.2 kilobase (kb) mRNA transcript. External application of 0.1% retinoic acid cream *in vivo* for 16 hours resulted in a 16-fold induction of CRABP-II, but not CRABP-I. CRABP-II mRNA, was also markedly increased (>15-fold) by retinoic acid treatment of fibroblast cultured from human skin, whereas no significant induction of CRABP-II mRNA was observed in human lung fibroblast. Human CRABP-II, but not CRABP-I mRNA was significantly induced by treatments which induce keratinocyte differentiation *in vitro*. Highly increased levels of CRABP-II RNA were found in psoriatic epidermis when compared to normal epidermis for 30 10 different patients. CRABP-I message levels were on the other hand very low or undetectable in both normal and psoriatic skin (data not presented in Figures). Previous studies of the induction of CRABP by natural and synthetic retinoids and their increased levels during keratinocyte differentiation are consistent with the expression of CRABP-II message seen in the present investigation, but not with the expression of CRABP-I. These

studies thus identify CRABP-II as the isoform likely to be expressed and regulated by RA in adult human skin.

Since mRNA isolated from skin biopsies used in this study is approximately 95% derived from keratinocytes (see Voorhees, J.J. et al., *Arch. Dermatol.* 105:695-701 (1972)), the majority of RA induction of CRABP-II seen *in vivo* (Figure 3 described below) cannot be explained by the presence of mRNA derived from dermal fibroblasts. This is in contrast to the reduction of CRABP-II transcripts observed in response to high concentrations of RA in keratinocytes *in vitro* (Figure 5C described below). When grown to postconfluence under the same conditions used herein, human keratinocytes undergo coordinate increases in involucrin content and transglutaminase activity, two key determinants of cornified envelope formation during terminal differentiation. Pillai, S. et al., *J. Cell. Physiol.* 143:294-302 (1990). Moreover, these events are accelerated and began to occur prior to confluence when the extracellular calcium concentrations were increased to 1.2 or 2.4 mM. Pillai, S. et al., *J. Cell. Physiol.* 143:294-302 (1990). CRABP-II mRNA levels responded to confluence and external calcium in an identical fashion (Figure 5 described below). These results strongly favor the concept that the stratified structure of the epidermis and/or the presence of dermis is an important determinant of CRABP-II regulation *in vivo*, and may help to account for the differential responsiveness of keratinocytes to RA *in vitro* and *in vivo*. The negative effect of high concentrations of RA on CRABP-II expression seen *in vitro* is similar to the effect of this compound on transglutaminase type I mRNA levels in cultured keratinocytes. See Floyd, E.E. et al., *Mol. Cell. Biol.* 9:4846-4851 (1989).

Whether RA induction of the human CRABP-II gene occurs at the level of transcription and whether this regulation is mediated by specific nuclear receptors remains to be investigated. It has been shown that human skin as well as cultured human skin fibroblasts express RAR- $\gamma$ . Krust, A. et al., *PNAS (USA)*. 86:5310-5314 (1989); and Elder, J.T. et al., *J. Invest. Dermatol.* 96:425-433 (1991). However, this does not explain the lack of RA induction of CRABP-II mRNA seen in cultured human lung fibroblasts and keratinocytes, also known to express RAR- $\gamma$ . Elder, J.T. et al., *J. Invest. Dermatol.* 96:425-433 (1991). If the CRABP-II gene is regulated by the RARs, additional tissue or cell-specific factors may be required for RA induction.

Members of the nuclear superfamily of receptors have been shown to interact with their responsive elements as dimers. Glass, C.K. et al., *Cell* 59:697-708 (1989). RARs have also been shown to interact with other members of this family (i.e., the thyroid hormone receptors) forming heterodimers. Glass, C.K. et al., *Cell* 59:697-708 (1989). One of the most exciting findings recently is that the thyroid hormone receptors require an



auxiliary protein (TRAP) to interact with the thyroid hormone responsive element in the growth hormone gene. O'Donnell, A.L. et al., *Mol. Endocrinol.* 5:94-99 (1991). TRAP apparently is forming a heterodimer with the thyroid hormone receptor on the responsive element. Such dimerization between nuclear receptors and other transcription factors could explain tissue-specific regulation. If there is a skin-specific factor forming a heterodimer with the RARs, that could explain why the CRABP-II gene is induced by RA in skin fibroblasts, but not lung fibroblasts.

In summary, we have demonstrated that CRABP-II is expressed in human skin *in vivo* and the CRABP-II gene appears regulated by RA in skin *in vivo* and in cultured skin fibroblasts *in vitro*. CRABP-II was not, however, induced by RA in cultured lung fibroblasts, demonstrating cell-specific regulation of this gene. CRABP-I, on the other hand, does not appear regulated by RA and is found at very low or undetectable levels in human skin *in vivo*, as well as in keratinocytes and fibroblasts. This suggests that CRABP-II may participate in a regulatory feedback mechanism to control the action of RA on cell differentiation in skin. The identification of human CRABPs, RARs and RXRs now allows studies, such as those described below, on interactions between members of these families in the complex molecular and cellular mechanisms of RA action.

The identification of the nucleic and amino acids sequences of human CRABP-I and CRABP-II provide the basis for a variety of recombinant products, including vectors carrying the human CRABP cDNA sequences and expression constructs cotransfected or infected with such vectors. For example, plasmid or viral vectors carrying human CRABP cDNA have been constructed and are used to cotransfect or coinfect receptor-deficient CV-1 monkey kidney cells. Reporter assay systems utilizing CV-1 recombinant expression constructs which include human CRABP cDNA, a reporter element containing a retinoid responsive element and a reporter gene, and preferably internal control sequences, such as generally described in Aström, A. et al., *Biochem. Biophys. Res. Comm.* 173:339-345 (1990), can also now be constructed. Chimeric receptor proteins such as those described in U.S. Patent No. 4,981,784 to Evans et al., can also be synthesized and utilized in reporter assays.

Assay systems employing human CRABP in conjunction with additional retinoid-binding proteins are also contemplated as within the scope of the invention. For example, as shown schematically in Figure 6, viral (e.g. SV40) vector carrying a human CRABP, a reporter plasmid carrying the retinoid responsive element (e.g. RRE<sub>3</sub>-tk-CAT), a viral (e.g. SV40) vector carrying a human RAR or RXR of interest and a  $\beta$ -galactosidase vector (pCH110) for an internal control are coinfectd or cotransfected into CV-1 cells. As shown

in Figure 6, the recombinant CV-1 construct is exposed to the binding ligand (e.g. retinoid) of interest. By ligand is meant a molecule which binds to the receptor binding protein and induces the expression of the gene of interest. In this assay system, induction of the reporter gene is used to assay for ligand binding to receptor protein. Absent any  
5 additional regulatory requirements, functional ligand would bind RAR or RXR, stimulating the expression (and translation) of the reporter gene (CAT). By including human CRABP in the system, the interaction between human CRABP and RAR or RXR or other binding receptors can be determined. For example, if CRABP sequesters RA in the cytoplasm, less ligand will reach the nucleus, thereby reducing RAR or RXR-mediated stimulation of the  
10 reporter gene.

The sequencing of human CRABP also allows the raising or production of antibodies or binding fragments, e.g. F'(ab), which can be used in immunoassays to further characterize binding or for diagnostic purposes and to monitor the course of patient treatment. For example, patient tissue can be assayed for the presence and levels of  
15 human CRABP to diagnose particular conditions where retinoids are implicated and to monitor the effectiveness of drugs and other treatments in altering patient levels of CRABP. CRABP purified directly from human tissue or cells, in culture or human CRABP produced synthetically or *ex vivo* will also provide CRABP for supplementation therapy where needed.

20 The gene for human cellular retinoic acid-binding protein II (CRABP-II) has been cloned and sequenced. It was isolated from a human placenta genomic library and is contained within one bacteriophage clone. The gene spans 6 kilobases and consists of four exons and three introns as do other members of the hydrophobic ligand binding protein gene family. Wei, L.N. et al., *DNA Cell Biol.* 9:471-478 (1990). The mouse CRABP-I  
25 gene has previously been cloned, demonstrating a different intron, but similar exon arrangement. Wei, L.N. et al., *DNA Cell Biol.* 9:471-478 (1990). One major transcription site was mapped to an A residue 137 nucleotides upstream of the ATG initiation codon. The sequence of the upstream region of the CRABP-II gene is rather GC rich and has a TATA box at -31, and several possible binding sites for transcription factors.

30 Of special interest is the presence of potential AP2 (CCC/GCA/GGGC) sites in the 5' flanking region. AP2 has been shown to be identical to the transcription factor KER1 which has been suggested to be generally involved in epidermal gene regulation. Leask, A. et al., *PNAS (USA)* 88: 7948-7952 (1991). CRABP-II is predominantly expressed in the skin of adult mice and we have recently demonstrated that CRABP-II, but not CRABP-I is  
35 expressed in human skin. Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990); Aström, A.

et al., *J. Biol. Chem.* 266:17662-17666 (1991). In addition, both AP2 and CRABP-II mRNA have been shown to be induced by RA. Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991); Lüscher, B. et al., *Genes Dev.* 3: 1507-1517 (1989). Whether AP2 is involved in skin-specific expression and RA induction of the CRABP-II gene remains to be determined.

5 As shown in Figure 8, the upstream region also contains a high affinity Sp1 binding site (GGGGCGGAGC) close to the TATA box, and two sequences (GCGGGGGCG) identical to Krox-24 binding sites. Kadonaga, J. T. et al., *Trends Biochem. Sci.* 11:20-23 (1986). Lemaire, P. et al., *Mol. Cell. Biol.* 10:3456-3467 (1990). Krox-24 is a member of the early responsive gene family, suggested to be involved in regulation of cell proliferation and

10 differentiation. Lemaire, P. et al., *Mol. Cell. Biol.* 10:3456-3467 (1990); Edwards, S.A. et al., *Dev. Biol.* 148:165-173 (1991). It was recently shown that Egr-1 Krox-24 was induced by RA in embryonal P19 cells. The induction of Egr-1 protein by RA in these cells, demonstrated a transient 5 fold increase, peaking around 30-60 minutes and returned to basic levels between 60-90 minutes. Thus, as shown in Figure 11, the induction of Egr-1

15 protein by RA just precedes CRABP-II gene transcription. However, the involvement of Egr-1 in RA induced transcriptional regulation of the CRABP-II gene in skin fibroblasts is unlikely, since Egr-1 was found to be induced by treatment of the cells with cycloheximide while CRABP-II gene expression in this study was found to be inhibited by this compound as demonstrated in Figures 12 and 13. Edwards, S.A. et al., *Dev. Biol.* 148:165-173 (1991).

20 The upstream region of the CRABP-II gene also contains a direct repeat (G/AGTTCA) spaced with one nucleotide with homology to the RARE found in the RAR- $\beta_2$  promoter, except that the RAR- $\beta_2$  RARE is spaced by five nucleotides. Umesono, K. et al., *Cell* 65:1255-1266 (1991). It was recently shown that two other members of the hydrophobic ligand-binding family of genes contain a RARE (CRBP-I) and a RXRE (CRBP-II) in their

25 promoters. Smith, W.C. et al., *EMBO J.* 10:2223-2230 (1991); Mangelsdorf, D.J. et al., *Cell* 66:555-561 (1991). Whether this direct repeat is functional remains to be determined. To determine whether RA induction of CRABP-II is transcriptional, nuclear run-on assays were performed. As can be seen in Figures 10 and 11, treatment of cultured human skin fibroblasts with RA resulted in a rapid transient 6-fold increase of transcription, followed by

30 a 6-fold induction of CRABP-II mRNA levels. Thus, the CRABP-II gene is mainly transcriptionally activated by RA. The CRABP-II mRNA was rapidly induced within 2-6 hours in cultured human skin fibroblasts by retinoic acid, reaching a plateau after 6 hours of treatment. However, removal of retinoic acid from the medium after 12 hours caused a sharp decline in CRABP-II mRNA levels. The rapid increase of CRABP-II message was

35 mainly due to an increased rate of transcription as determined by the nuclear run-on

experiments as shown in Figure 10. Increased transcription could be detected as early as 1 hour after addition of RA, peaked at 2 hours and returned to basal levels within 6 hours. In addition, both the accumulation of message, as shown in Figure 12, and the induction of transcription, as shown in Figure 13, by RA was inhibited by cycloheximide, suggesting  
5 that the CRABP-II gene is transcriptionally regulated by a newly synthesized protein. Once transcription had occurred the message reached a plateau and did not decline until RA was removed from the medium. On-going protein synthesis was required for the transient increase in transcription, since the induction was blocked by cycloheximide. It is very unlikely that the decrease of CRABP-II mRNA seen was a result of reduced transcription  
10 of the gene, since the RA induction of transcription was transient and back to control levels within 6 hours. A more likely explanation would be that RA was involved in stabilization of the CRABP-II message. The CRABP-II mRNA is likely to be unstable, since it contains AU rich sequences in the 3' untranslated region. Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990); Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991). It has been  
15 shown that many transiently expressed genes, including lymphokine genes, c-myc, and c-fos contain AU rich sequences in their 3' untranslated regions, and that the presence of these sequences correlates with rapid mRNA degradation. Cleveland, D.W. et al., *New Biol.* 1:121-126. Mechanisms by which CRABP-II mRNA stabilization could occur, may involve the production of a factor after treatment of the cells with RA, or interaction of RA  
20 with a preexisting factor, stabilizing the message. Since cycloheximide blocked the RA induced transcription of the gene and as a consequence, the induction of the message, the possibilities were not distinguishable. It has been demonstrated that the human CRABP-II gene is transiently transcriptionally induced by RA in human skin fibroblasts, and that this induction is dependent on on-going protein synthesis. The early induction of  
25 transcription is followed by a rapid increase in CRABP-II message levels that does not decrease until RA is removed from the medium, suggesting RA dependent mRNA stabilization.

It will be appreciated that the nucleotide and amino acid sequences of the present invention can include some variation from the sequences represented by and  
30 complementary to the sequences set forth in the Sequence Listing, but must be substantially represented by or complementary to those set forth therein. By "substantially represented by" or "substantially complementary to" is meant that any variation therein does not impair the functionality of the sequence to any significant degree. As used herein, A represents adenine; T represents thymine; G represents guanine; and C  
35 represents cytosine; except where otherwise indicated.

## SPECIFIC EXAMPLES

### SPECIFIC EXAMPLE 1. Cloning and Sequencing of Human CRABP

#### MATERIALS AND METHODS

##### *Cloning of CRABP from human skin RNA by Polymerase Chain Reaction (PCR)*

5        Total RNA was isolated from human keratome biopsies as described in Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990) and cDNA was synthesized by reverse transcription as described in Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). Degenerate primers derived from mouse and bovine CRABP-I were designed so as to amplify the coding  
10    region. XbaI and BamHI sites were contained in the forward and reverse primers, respectively, which are shown in Figure 1A.

      The cDNA was used as a template for PCR (2 minutes denaturation at 92°C, 2 minutes, annealing at 42°C, 2 minutes amplification at 72°C and final extension for 10 minutes). After 40 cycles, the length of the amplified DNA was determined on a 1.5%  
15    agarose gel. The amplified 436-bp region of CRABP was isolated from the gel, subcloned into Bluescript phagemid and sequenced as described below.

##### *Screening and sequencing of cDNA clones*

      Poly A+ RNA was prepared from human skin as described in Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990) and used to prepare a cDNA library in Lambda ZapII  
20    (Stratagene Inc., La Jolla, CA). The library contained  $1.0 \times 10^7$  primary recombinants. The 436-bp PCR product was labeled by random hexamer priming (Boehringer Mannheim, Indianapolis, IN) and used to screen the adult human whole skin cDNA library and a human skin fibroblast cDNA library in  $\lambda$ gt11 (Clontech, Palo Alto, CA). Duplicate nitrocellulose filters were hybridized for 16 hours in 50% formamide containing 5 x SSC (1  
25    x SSC = 150 mM NaCl, 15 mM sodium citrate), 1 x Denhardt's (0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone), 0.1% SDS (sodium dodecyl sulfate) and 200  $\mu$ g/ml tRNA. The filters were washed two times for 20 minutes in 0.2 x SSC, 0.2% SDS and one time for 20 minutes in 0.2 x SSC, 0.1% SDS at 55°C. Two positive clones were isolated from the skin library and five clones from the skin fibroblast library. The clones  
30    from the skin library were rescued, while the clones from the fibroblast library were subcloned into Bluescript phagemids (Stratagene Inc., La Jolla, CA).

      DNA sequence analysis was performed on both strands by dideoxy chain termination as generally described in Sanger, F. et al., *PNAS (USA)* 74:5463-5467 (1977), using modified T7 polymerase (Sequenase, U.S. Biochemical Corp.) and synthetic  
35    oligonucleotides.



**Cell culture**

Primary cultures of normal human keratinocytes were prepared as described in Boyce, S.T. et al., in *In Vitro Models for Cancer Research* (Weber, M.M., and Sekely, L.I., eds) Vol. 3, pp. 245-274. CRC Press, Boca Raton, FL (1986). Subcultures were expanded  
5 in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA). Human dermal fibroblast cultures were prepared from punch biopsies of buttock skin (see Harper, R.A. et al., *Science* 204:526-527 (1979)) and propagated in modified McCoy's 5A medium containing 10% calf serum. Human lung fibroblasts were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and grown in the same medium.

**10 Northern analysis of mRNA**

RNA was isolated from keratome biopsies and cultured cells by guanidinium isothiocyanate lysis and ultracentrifugation as previously described in Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990). For studies involving retinoic acid treatment, 0.1% RA cream (Retin-A, Ortho Pharmaceutical Corp. Raritan, NJ) was applied once to skin and  
15 maintained under plastic wrap for 4 hours to 96 hours prior to biopsy. Adjacent sites were treated with Retin-A vehicle or left untreated. After 4 hours, 12 hours, 16 hours or 96 hours, keratome biopsies were obtained and used for RNA isolation.

RNA concentrations were determined by absorbance at 260 nm and verified by nondenaturing agarose gel electrophoresis and ethidium bromide staining as described  
20 in Thompson, C.B. et al., *Nature* 314:363-366 (1985). Equal quantities of total RNA were electrophoretically separated in 1% formaldehyde-agarose gels containing 0.5 µg/ml ethidium bromide and transferred to derivatized nylon membranes (Zeta-Probe, BioRad, Richmond, CA) as described in Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990) and Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory,  
25 Cold Spring Harbor, NY (1982). Filters were baked 2 hours at 80°C in vacuo, then prehybridized for 2-4 hours at 42°C in 50% formamide, 5 x SSC, 50 mM sodium phosphate, pH 7.0, 1 x Denhardt's solution, 250 µg/ml yeast tRNA, 100 µg/ml sonicated herring sperm DNA and 1% SDS. Hybridization was carried out for 18-24 hours at 42°C in the same buffer containing 10% dextran sulfate. Filters were washed twice for 10 minutes in 2 x  
30 SSC, 0.1% SDS at room temperature, then twice for 20 minutes in 0.1 x SSC, 0.1% SDS at 56°C. Autoradiography was performed using intensifying screens at -70°C. Filters were stripped by boiling 2 x 10 minutes in 0.1 x SSC, 0.5% SDS. Hybridization probes were prepared by random priming (see Feinberg, A.P. et al., *Anal. Biochem.* 132:6-13 (1983)) of low melting agarose-purified insert fragments from the human CRABP-I PCR product,



the CRABP-I cDNA ( $\lambda$ f1.1) and rat cyclophilin. See Danielson, P.E. et al., *DNA* 7:261-267 (1988).

Autoradiograms were quantitated using a laser densitometer (LKB Model 2202) coupled to a Hewlett-Packard 3390 A integrator. Only exposures which were in the linear range of densitometric response were used. When appropriate, autoradiographic intensities were normalized to cyclophilin. See Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990). Statistical analysis of data was performed by one way analysis of variance using Scheffes correction for multiple comparison and a two-tailed hypothesis.

## RESULTS

### 10 *Cloning of human CRABP-I and CRABP-II*

Using PCR and degenerate primers derived from bovine and mouse CRABP-I, a 436 base pair (bp) product was obtained, subcloned and sequenced. The predicted amino acid sequence of the PCR product was found to be 99.3% homologous to the mouse and bovine CRABP-I sequences. See Nilsson, M.H.L. et al., *Eur. J. Biochem.* 173:45-51 (1988); and Stoner, C.M. et al., *Cancer Res.* 49:1497-1504 (1989). The PCR product was used to screen a human skin library and a human skin fibroblast library. Five clones were isolated from the skin fibroblast library ( $\lambda$ f1.1,  $\lambda$ f3.1,  $\lambda$ f5.1,  $\lambda$ f5.4 and  $\lambda$ f5.6). Two clones ( $\lambda$ f1.1,  $\lambda$ f3.1) were sequenced and found to be identical. The predicted amino acid sequences of these clones were found to be 77.4% similar to human CRABP-I and 93.5 % similar to the recently cloned mouse CRABP-I. See Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990). Because of the high homology to the mouse CRABP-II, clone  $\lambda$ f1.1 was designated as human CRABP-II. A third clone  $\lambda$ f5.1 was partially sequenced and found to be a shorter clone, with a sequence identical to human CRABP-II. No CRABP-I clones were isolated from the skin fibroblast library. Two clones were isolated from the skin library and found to contain shorter inserts, one with a sequence identical to human CRABP-II ( $\lambda$ s2.1) and one with a sequence identical to human CRABP-II ( $\lambda$ s3.1).

SEQ ID NOS. 1 and 2 in the Sequence Listing represent the cDNA nucleotide and predicted amino acid sequences of human CRABP-II. The translation initiation site was assigned to the first methionine codon corresponding to nucleotides 99-101. An open-reading frame of 138 amino acids was found, predicting a polypeptide of  $M_r$  15,693. The 3' untranslated region was found to contain a polyadenylation signal (ATTAAA) and a poly(A) tract of 1 ( $\lambda$ f1.1) to 25 ( $\lambda$ f5.1). SEQ ID NOS. 3 and 4 represent the cDNA nucleotide and predicted amino acid sequence of CRABP-I respectively.

**Comparison of amino acid sequences of CRABP**

The amino acid sequence comparison of CRABP-I and CRABP-II is presented in Figure 2. Panel A is a comparison of the amino acid sequences for human (h) and mouse (m) CRABP. The predicted amino acid sequences of mouse and human CRABP-I and mouse CRABP-II were aligned with human CRABP-II. Dashes (—) represent identity to human CRABP-II. One gap as indicated by an asterisk (\*) was introduced in the human and mouse CRABP-I sequences for maximum alignment. Panel B of Figure 2 is the sequence comparison of the NH<sub>2</sub>-terminal ends of human (h), mouse (m), rat (r), CRABP-I and CRABP-II. Residues dissimilar to human CRABP-II are boxed.

10 The amino acid sequence comparison presented in Figure 2, Panel A, reveals a 73.7% overall degree of identity between mouse CRABP-I (Stoner, C.M. et al., *Cancer Res.* 49:1497-1504 (1989); Giguere, V. et al., *PNAS (USA)* 87:6233-6237 (1990)) and between human CRABP-I and human CRABP-II. Human CRABP-I and mouse CRABP-I displayed an overall identity of 99.3%, with a single amino acid substitution (amino acid residue 86, Ala instead of Pro), while human CRABP-II and mouse CRABP-II were 93.5% identical. Human and mouse CRABP-II displayed 9 amino acid differences with the following amino acids in the human sequence: residue 19 - Leu, 22 - Val, 27 - Val, 29 - Leu, 48 - Gly, 68 - Val, 91 - Glu, 99 - Lys and 111 - Thr. In 6 of the 9 amino acid differences seen between human CRABP-II and mouse CRABP-II (residues 19, 29, 48, 68, 91 and 111), the human sequence was identical to CRABP-I.

20 The NH<sub>2</sub>-terminal amino acid sequences of two CRABPs from neonatal rat (Bailey, J.S. et al., *J. Biol. Chem.* 263:9326-9332 (1988)) and chicken embryos (Kitamoto, T. et al., *Biochem. Biophys. Res. Comm.* 157:1302-1308 (1988)) have been reported and were also compared to human CRABP-I and CRABP-II. As shown in Figure 2, Panel B, there were no amino acid differences between the NH<sub>2</sub>-terminal regions of rat, chicken, mouse and human CRABP-I, whereas several differences appeared between the CRABP-IIs.

**SPECIFIC EXAMPLE 2. Expression of Human CRABP****Expression of human CRABP in human skin in vivo**

Volunteers were treated with 0.1% RA cream or control treatments under occlusion for various time intervals. Keratome biopsies consisting mostly of epidermis (see Voorhees, J.J. et al., *Arch. Dermatol.* 105:695-701 (1972)) were obtained, and used to prepare total RNA which was subsequently analyzed by blot hybridization. The autoradiograms of the RNA blots derived from three individuals are shown in Figure 3. Total RNA (40 µg per lane) was hybridized against the human CRABP-II or cyclophilin cDNA probes as described above. Each volunteer was treated topically for 16 hours prior

to biopsy as follows: (a) no treatment, (b) RA vehicle or (c) 0.1% RA cream in RA vehicle under occlusion with plastic wrap. Mobilities of ribosomal RNAs are indicated to the left of the blots.

CRABP-II transcripts were detectable in untreated skin as well as skin treated with vehicle, as shown in Figure 3. CRABP-II transcripts were markedly (16.1-fold) and significantly ( $p < 0.004$ ,  $n = 4$ ) induced in RA treated relative to untreated skin. Similar, albeit less marked, inductions were observed after 4 days of RA treatment ( $8.3 \pm 2.9$ -fold,  $n = 6$ ). Induction did not occur after 4 hours, but was evident after 12 hours of treatment. Consistent with our ability to amplify CRABP-I from human skin RNA, faint CRABP-I probe hybridization was observed in some but not all blots of human skin RNA samples after prolonged autoradiographic exposure (data not presented in Figures). However, CRABP-I transcripts were usually undetectable under exposure conditions sufficiently sensitive to detect single copy DNA sequences from 10  $\mu$ g human genomic DNA. CRABP-I and CRABP-II cDNA probes detected distinct band patterns using genomic DNA digested with BamHI, EcoRI, HindIII and PstI, demonstrating the specificity of these probes under our hybridization conditions (data not presented in Figures).

#### ***Expression of CRABP in human fibroblasts***

Treatment of human dermal fibroblasts with RA resulted in a marked (approximately 15-fold) and significant ( $P < 0.04$ ) induction of human CRABP-II mRNA after treatment of five independent dermal fibroblast strains with  $3 \times 10^{-7}$  or  $3 \times 10^{-6}$  M RA for 24 or 48 hours as shown in Figure 4. Results shown are derived from nine independent experiments involving five dermal fibroblast lines prepared from three individuals as described above and three diploid human lung fibroblast lines (LL47, CCD-18Lu, and CCD-16Lu). RNA blot hybridizations (20  $\mu$ g total RNA/lane) were quantitated by laser densitometry and normalized to the control gene cyclophilin, as described in Elder, J. T. et al., *J. Invest. Dermatol.* 94:19-25 (1990). At confluence, medium was changed and cells were treated with RA dissolved in dimethyl sulfoxide at the concentrations and for the times indicated beneath the Figure. Data are expressed as fold induction  $\pm$  SEM, relative to the average of duplicate dishes treated with dimethyl sulfoxide alone for 4 hours \* $p < 0.05$ , \*\* $p < 0.005$ . The relevant autoradiographic bands from two representative experiments comparing dermal and lung fibroblasts are shown in the inset.

As shown in Figure 4, CRABP-II mRNA was not significantly induced by RA in three strains of human lung fibroblasts, suggesting that this response may be tissue specific. Induction of human CRABP-II transcripts by RA was dose-dependent over the range of  $3 \times 10^{-10}$  to  $3 \times 10^{-7}$  M RA (data not presented in Figures). In contrast, CRABP-I transcripts

were undetectable in dermal and lung fibroblasts and were not induced by RA, whereas genomic DNA blots hybridized in parallel were positive.

#### **Expression of CRABP in human keratinocytes**

As shown in Figure 5, CRABP-II mRNA was markedly and significantly induced in  
5 cultured adult human keratinocytes when cells reached confluence, and remained elevated  
in the postconfluent state. Figure 5A summarizes the results obtained for seven  
independent keratinocyte strains. Third passage normal adult human keratinocytes were  
grown in KGM containing 0.15 mM  $\text{CaCl}_2$ , and medium was changed every other day. At  
the indicated number of days pre- or post-confluence, total RNA was prepared and  
10 analyzed for human CRABP-II mRNA by blot hybridization and densitometry. Data are  
expressed as percent maximal expression for any given strain of keratinocytes, as the  
absolute level of human CRABP-II mRNA was variable from strain to strain. Error bars  
represent  $\pm$  SEM. \*\* $p < .005$ , \*\*\* $p < 0.0005$  relative to cells at 2 days preconfluence.

CRABP-II mRNA was also markedly induced in subconfluent cultures by raising the  
15 calcium concentration in the medium from 0.15 mM to 2 mM, as illustrated for a  
representative strain of keratinocytes in Figure 5B. At 20-30% confluence, the medium was  
changed to KGM or KGM containing 2 mM  $\text{CaCl}_2$  in the presence or absence of  $3 \times 10^{-9}$   
M RA and maintained for the indicated number of days, with medium change every other  
day. Mobilities of 28S and 18S ribosomal RNAs are indicated to the left. Figure 5C shows  
20 the effects of prolonged treatment with high concentrations of RA on CRABP-II mRNA  
levels. The same experiment described for Figure 5B was conducted above, except that  
cells were treated with or without  $3 \times 10^{-6}$  M RA for 2 to 5 days, as indicated above the  
autoradiograms. Mobilities of 28 and 18S ribosomal RNAs are indicated to the left.  
Figures 5A and B show the results representative of four independent experiments.

25 As shown in Figure 5B, treatment of keratinocytes for prolonged periods of time  
with low concentrations of RA ( $3 \times 10^{-9}$  M) had no detectable effect on CRABP-II mRNA  
levels. However, as shown in Figure 5C, prolonged treatment of subconfluent  
keratinocytes with high concentrations of RA ( $3 \times 10^{-6}$  M) reduced CRABP-II mRNA to  
undetectable levels. Hybridization of the same blot against CRABP-I prior to its  
30 hybridization against CRABP-II failed to detect CRABP-I transcripts, whereas genomic DNA  
blots hybridized in parallel were positive (data not presented in Figures).

#### **SPECIFIC EXAMPLE 3. Alternate Cloning Scheme**

The CRABP-II cDNA probe was cloned by PCR from retinoic acid (RA) treated  
human skin using the CRABP-II degenerate primers shown in Figure 1B, derived from  
35 mouse CRABP-II mRNA sequence. See Giguere, V. et al., *PNAS (USA)* 87:6233-6237

(1990). BamHI restriction sites were included in the forward and reverse primers to aid in subsequent cloning.

RA-treated human skin was used as the source of RNA for reverse transcription and PCR amplification. Total RNA was extracted from epidermal keratomes as described in Elder, J.T. et al., *J. Invest. Dermatol.* 94:19-25 (1990), and reverse transcribed as described in Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). During each PCR cycle, samples were heated to 94°C in 1 minute and maintained for 30 seconds, cooled to 50°C for primer annealing, and then heated in 1 minute duration to 72°C and kept at that temperature for a further 30 seconds to allow template extension. The PCR product was further purified (Geneclean, BIO 101 Inc.), digested with BamHI and cloned into BamHI digested PGEM 3Z plasmid (Promega Inc., Madison, WI). The PCR fragment was initially identified as CRABP-II by digestion with PstI restriction enzyme that cleaved the DNA at the same position as that described in the mouse CRABP-II mRNA. The clone was sequenced and found to represent the major portion of the mature human analogue of the mouse CRABP-II. The cDNA probe was hybridized to human RNA prepared from skin of human volunteers topically treated with RA for 4 and 12 hours. Induction of CRABP-II mRNA was observed in skin which had been RA-treated for 12 hours, but not skin treated for only 4 hours (data not illustrated in Figures).

#### 20 **SPECIFIC EXAMPLE 4. CRABP Mammalian Expression Vectors and Constructs**

CRABP-I was excised from the Bluescript phagemid (Stratagene) cloning vector and inserted between the XbaI and BamHI sites of the eukaryotic expression vector pSVL (Pharmacia) in forward orientation (pSVLCRABP-I). CRABP-II was excised from the Bluescript phagemid (Stratagene) cloning vector and inserted into the EcoRI site of the eukaryotic expression vector pSG5 (Stratagene) in forward (pSG5CRABP-II) or reverse orientation (pSG5CRABP-IIAS). The orientation of the insert was determined by restriction analysis.

CV-1 cells are grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum. CV-1 are monkey kidney cells derived from the CV-1 cell line which do not express T cell antigen. The day before transfection, cells are seeded on tissue culture dishes in DMEM containing 10% charcoal treated fetal calf serum (ChFCS). Cells are cotransfected using the calcium phosphate co-precipitation technique essentially as described in Rosenthal, N., *Meth. Enzymol.* 152:704-720 (1987) with CRABP-I (pSVLCRABP-I) or CRABP-II (pSG5CRABP-II) expression vector. 24 hours after transfection, cells are trypsinized and suspended in medium, pelleted and washed once with 40mM Tris-C1, pH

7.6 containing 150 mM NaCl and 1 mM EDTA. A cytosolic fraction is prepared on cell lysates by centrifugation at 100,000 x g for 1 hour.

#### **SPECIFIC EXAMPLE 5. Bacterial Production of Human CRABP**

For CRABP-I, the nucleotide sequence of CRABP-I is changed at position 9 (T to G) and 13 (C to T) to create a *Stu*I site, using synthetic oligonucleotides and the polymerase chain reaction. For CRABP-II, the nucleotide sequence of CRABP-II is changed at position 100 (T to G) and 104 (C to T) to create a *Stu*I site, using synthetic oligonucleotides and the polymerase chain reaction. The mutated CRABP cDNAs are then cut with *Stu*I and ligated into the *Stu*I and *Eco*RI sites of the bacterial expression vector pMAL-c (New England Biolabs). Bacteria is transformed and the maltose-binding protein (MBP)-CRABP fusion proteins are expressed in large quantities. The MBP-CRABP fusion protein is purified by affinity chromatography on any amylose column (New England Biolabs). CRABP lacking the first methionine is released from MBP by digestion with factor Xa (New England Biolabs) and purified from MBP by a second passage over an amylose column.

Spectrofluorimetric methods are used to study the affinities and binding stoichiometries of purified human CRABP-I and CRABP-II for a variety of ligands. The capacity of ligands to bind to CRABP-I or CRABP-II is assessed by monitoring their ability to quench the native fluorescence of this protein.

#### **20 SPECIFIC EXAMPLE 6. Production of Antibodies**

Peptides corresponding to amino acids 94 to 104 in the CRABP-I and CRABP-II proteins have been synthesized using the multiple antigenic peptide method as generally described in Posnett, D.N. et al., *Meth. Enzymol.* 178:739-746 (1989), eliminating the need for conjugation to a carrier protein. This region of the two CRABPs has a low homology and is most probably situated on the outside of the proteins based on hydrophobicity and surface probability calculations.

The peptides were injected into chickens for production of egg IgY. The resulting antibodies, if not specific, are adsorbed to peptides (CRABP-I antibodies to CRABP-II peptides and the reverse) immobilized on a sepharose gel to enhance specificity. Once monospecific antibodies are obtained, the expression and regulation of CRABP-I and CRABP-II proteins in human skin and skin cells is examined quantitatively by Western blot analysis and semi-quantitatively by immunocytochemistry. The pattern of expression and regulation of CRABP-II (and CRABP-I) by RA in normal and psoriatic skin will provide insight into the function of CRABPs.



Antibodies to CRABP-I and CRABP-II, or binding fragments (e.g. F'(ab)) thereof, and the purified proteins obtained as described above can be used to monitor levels of CRABP-I and CRABP-II in normal and pathological states by using immunological techniques known to those skilled in the art. For example, patient skin tissue can be  
5 assayed with antibody specific for human CRABP-I or CRABP-II to determine the presence and levels of CRABP by ELISA, Western blot analysis or immunochemistry essentially as described in Busch, C. et al., *Meth. Enzymol.* 189:315-324 (1990).

#### **SPECIFIC EXAMPLE 7. Reporter Assay System**

CV-1 cells are grown in Dulbecco's modified eagles medium (DMEM) containing  
10 10% fetal calf serum. The day before transfection, cells are seeded on tissue cultured dishes in DMEM containing 10% charcoal treated fetal calf serum (CHFCS). Cells are cotransfected using the calcium phosphate co-precipitation technique with 0.6 mg of human retinoic acid receptor (hRAR) expression vectors (hRAR $\alpha$ 0, hRAR $\beta$ 0 or hRAR $\lambda$ 0), a reporter plasmid and a  $\beta$ -galactosidase expression vector (pCH110, Pharmacia) used as  
15 an internal control to normalize for variations in transfection efficiency essentially as described in Aström, A. et al., *Biochem. Biophys. Res. Comm.* 173:339-345 (1990). Cells are also cotransfected with CRABP-I (pSVLCRABP-I) or CRABP-II (pSG5CRABP-II) expression vectors of pSVL (Pharmacia) as a control. The reporter plasmid (TRE)<sub>3</sub>-tk-CAT is constructed by ligating synthetic oligonucleotides encoding three palindromic thyroid  
20 hormone responsive elements (TRE) ((TCAGGTCATGACCTGA)<sub>3</sub>) flanked by HindIII and BamHI sites on the 5' and 3' ends respectively and cloned into the HindIII - BamHI cloning sites of the plasmid pBLCAT2. 24 hours after transfection, cells are washed once with DMEM, and medium (DMEM, 10%ChFCS) containing different concentrations of ligands are added to the cells. 24 hours later the cells are trypsinized and suspended in medium,  
25 pelleted and washed once with 40mM Tris-Cl, pH 7.6 containing 150mM NaCl and 1mM EDTA. Cell lysates are prepared by three consecutive freeze-thaw cycles and  $\beta$ -galactosidase and CAT-activities are determined by a xylene extraction method. The effect of coexpression of CRABP-I or CRABP-II on RAR transcriptional activation for different ligands can now be determined as described below.

30 An alternative reporter assay in which a recombinant adenovirus system is used to coinfect cells in culture may also be employed to measure transcriptional activation by retinoids essentially as described in Shih, E. et al., *Mol. Endocrinol.* 5:300-309 (1991). In such a system, two mutually dependent viruses, one containing a receptor transcription unit and the second containing a gene responsive element are coinfecting into receptor-  
35 deficient cells such as CV-1. For example, two mutually dependent adenoviruses, one

containing a human glucocorticoid receptor transcription unit and the other a glucocorticoid responsive element linked to the firefly luciferase gene, or one containing rat thyroid hormone receptor  $\alpha$  and the other the luciferase gene, can be utilized in the practice of the assay. Hormone-induced transcription is then quantitated after infection  
5 from cells coinfecting with the complementary virus pair.

#### **SPECIFIC EXAMPLE 8. Ligand Binding Assays**

Since the amino-acid homology between CRABP-I and CRABP-II is only 77%, it is important to determine the ligand binding properties of these two proteins. There are several reports on the lack of correlation between biological activity and affinity of retinoids  
10 to CRABP. Darmon, M. et al., *Skin Pharmacol.* 1:161-175 (1988). Most of these studies have used extracts from rat testis containing mostly rat CRABP-I as the source of CRABP. In contrast, we are expressing the CRABP-I and CRABP-II cDNAs can now be expressed in mammalian cells.

Availability of cloned human CRABP-I and II thus improves previous systems for  
15 identification of tissue-specific ligands. This also makes identification of ligands interacting with CRABP possible, in addition to nuclear receptors. Ligands for testing include, but are not limited to dihydro-RA, RA and its metabolites, 4-hydroxy-RA, 4-oxo-RA, and 5,6-epoxy-RA as well as compounds previously reported not to bind to CRABP, e.g. CD-394. Darmon, M. et al., *Skin Pharmacol.* 1:161-175 (1988). Availability of CRABP amino-acid  
20 sequences or proteins provide the means to select more tissue-specific drugs for repair of photoaging skin, psoriasis, acne, skin cancer, leukemia, diseases of keratinization, osteoporosis, rheumatoid sclerosis, and other conditions.

Soluble protein from CV-1 cells transfected with CRABP-I (pSVLCRABP-I) or CRABP-II (pSG5CRABP-II) expression vectors obtained as described above is incubated  
25 with [ $^3$ H] retinoic acid at 4°C overnight. Free ligand is separated from bound ligand by size fractionation on a GF 250 column (DuPont Pharmaceuticals) connected to a FPLC system (Pharmacia). The amount of specific binding is determined by incubating samples with an excess of cold retinoic acid. The affinity of CRABP-II for retinoic acid can be determined by titrating with increasing amounts of [ $^3$ H] Retinoic acid.

30 The relative affinity for other ligands is determined in competition assays in the same system. A fixed amount of CRABP-I or [ $^3$ H] retinoic acid is incubated with soluble protein from CV-1 cells expressing CRABP-II together with increasing amounts of other possible ligands.

**SPECIFIC EXAMPLE 9. Hybridization Assay**

Availability of cloned human CRABP-I and CRABP-II cDNAs makes it possible to determine the relative expression of these two genes in normal and pathological states. For example, RNA is isolated from tissue biopsies and cultured cells by guanidinium isothiocyanate lysis and ultracentrifugation as previously described in Elder, et al., *J. Invest. Dermatol.* 94:19-25 (1990). Equal quantities of total RNA can be separated on 1% formaldehyde-agarose gels and transferred to nylon membranes. After baking for 2 hours at 80°C, filters can be hybridized to agarose-purified CRABP-I or CRABP-II cDNAs labeled by random priming. The amount of CRABP mRNA in a tissue can be determined after quantitation of autoradiograms by laser-densitometry as described (Elder, et al., *J. Invest. Dermatol.* 94:19-25). It will be appreciated that CRABP-I and II oligonucleotides (typically 8-15 residues long) may also be utilized as probes in hybridization assays if of a sufficient length to bind complementary sequences.

**SPECIFIC EXAMPLE 10. Examination of Function of Human CRABP****15 Receptor assay**

The most important and difficult issue to resolve regarding the CRABPs is their function. It has been suggested that CRABP transports RA to the nucleus (Takase, S. et al., *Arch. Biochem. Biophys.* 247:328-334 (1986)) or that CRABP remains in the cytoplasm, thereby preventing RA from moving to the nucleus. Maden, M. et al., *Nature* 335:733-735 (1988). One way of addressing this issue is to express increasing amounts of CRABP-I and CRABP-II in CV-1 cells together with the RARs and a reporter gene containing a retinoic acid responsive element, as described above. If CRABP transports RA to the nucleus an enhancement of reporter gene activity at low concentrations of RA may be seen. On the other hand, if CRABP sequesters RA a decrease in response will be seen.

**25 Overexpression**

CRABP-I and CRABP-II is overexpressed in fibroblasts and keratinocytes. Cells will be transfected with CRABP expression vectors (pSVLCRABP-I or pSG5CRABP-II) as described above, using Lipofectin (BRL) essentially as described by the manufacturer. In fibroblasts the effect of CRABP overexpression on induction of the RAR- $\beta$  gene by RA is studied. It has been shown that RAR- $\beta$  mRNA is induced by RA in skin fibroblasts, and it is known that this gene is directly regulated by the RARs. See DeThe, H. et al., *Nature* 343:177-180 (1990). The RAR- $\beta$  gene acts as an "endogenous reporter gene" for RAR induction. In proliferating keratinocytes the effect of overexpression of CRABPs on markers of differentiation is studied.

**Translation blocking**

Translation of CRABP-II is blocked in fibroblasts and keratinocytes by transfecting the cells with an expression vector construct having the CRABP-II cDNA in a reverse orientation. This produces an anti-sense mRNA that is able to hybridize to the  
5 endogenous CRABP-II mRNA, thereby blocking translation. The effect of antisense expression in fibroblasts and keratinocytes is studied the same way as described for overexpression.

**Effect of CRABP on RA metabolism**

The involvement of the cellular retinol binding protein (CRBP) in delivering retinol  
10 to the appropriate metabolic enzyme in rat liver microsomes has been described. Ong, D.E. et al., *J. Biol. Chem.* 263:5789-5796 (1988). Since the CRABPs belong to the same family of binding protein as CRBP, it is possible that they also are involved in the metabolism of their ligands. RA is metabolized by the cytochrome P-450 monooxygenase system present in the endoplasmic reticulum. Bossche, H. et al., *Skin Pharmacol.* 1:176-  
15 185 (1988). CRABP may function by increasing the interaction of RA with cytochrome P-450. Another possibility is that RA is not the natural ligand for CRABP-II, but rather one of the metabolites. If CRABP-II for example, binds 4-hydroxy-RA (as determined by ligand-binding studies) with a higher affinity than RA, it is possible that CRABP-II increases cytochrome P-450 mediated metabolism by decreasing product concentration. Whether  
20 the CRABPs are involved in RA metabolism or not is tested by incubating skin microsomes with increasing amounts of RA and of expressed and purified CRABP-I or CRABP-II. The effects of CRABPs on RA metabolism is assayed by HPLC.

**SPECIFIC EXAMPLE 11. Cloning and Sequencing of Human CRABP-II Gene and Transcription Studies****25 MATERIALS AND METHODS****Cloning of the human CRABP-II gene**

To isolate the CRABP-II gene, approximately  $5 \times 10^5$  recombinants from a human placenta genomic DNA library in Lambda FIX II (Stratagene Inc., LaJolla, CA) was screened using the human CRABP-II cDNA as a probe. Aström, A. et al., *J. Biol. Chem.* 266:17662-  
30 17666 (1991). One positive clone was isolated, purified and restriction mapped. Parts of the insert were subcloned into Bluescript phagemid SK (Stratagene Inc., LaJolla, CA). The sequence of the gene was determined on both strands after subcloning into M13 vectors by dideoxy chain termination using modified T7 polymerase (Sequenase, U.S. Biochemical Corp.) and synthetic oligonucleotides. Sanger, F. et al., *PNAS (USA)* 74:5463-5467 (1977).  
35 Exon positions were determined by restriction mapping and sequencing.

**Cell culture**

Human dermal fibroblast cultures were prepared from punch biopsies of buttock skin and propagated in Dulbecco's modified Eagle's medium containing 10% calf serum. Harper, R.A. et al., *Science* 204:526-527 (1979).

**5 Northern analysis of mRNA**

RNA was isolated from cultured human skin fibroblasts by guanidinium isothiocyanate lysis and ultracentrifugation as previously described. Elder, J.T. et al., *J. Invest. Dermatol* 94:19-25 (1990). RNA concentrations were determined by absorbance at 260 nm and equal quantities of total RNA were electrophoretically separated in 1% formaldehyde-agarose gels containing 0.5  $\mu$ g/ml ethidium bromide. The RNA was transferred to nylon membranes (Zeta-Probe, BioRad, Richmond, CA) as described. Elder, J.T. et al., *J. Invest. Dermatol* 94:19-25 (1990); Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1982). Filters were basked 2 hours at 80°C *in vacuo*, then prehybridized for 2-4 hours at 42°C in 50% formamide, 5 x SSC (1 x SSC = 150mM NaCl, 15 mM sodium citrate), 50 mM sodium phosphate, pH 7.0, 1 x Denhardt's solution, 250  $\mu$ g/ml yeast tRNA, 100  $\mu$ g/ml sonicated herring sperm DNA and 1% SDS. Hybridization was carried out for 18-24 hours at 42°C in the same buffer. Filters were washed once in 0.2 x SSC, 0.1% SDS at room temperature, then twice for 20 min in 0.2 x SSC, 0.1% SDS at 56°C, and finally once for 20 min in 0.1 x SSC, 0.1% SDS at 56°C. Autoradiography was performed using intensifying screens at -70°C. Filters were stripped by boiling 2 x 10 min in 0.1 x SSC, 0.1% SDS. Hybridization probes were prepared by random priming (Boehringer Mannheim) of purified insert fragments from human CRABP-II cDNA ( $\lambda$ f1.1) rat cyclophilin and RAR- $\gamma$ , Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991); Danielson, P.E. et al., *DNA* 7:261-267; Elder, J.T. et al., *J. Invest. Dermatol* 96:425-433 (1991). Quantitation of mRNA levels were performed using a phosphorimager (Molecular Dynamics).

**Primer extension analysis**

RNA was isolated from untreated and RA treated skin fibroblasts as described. Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991). A synthetic oligonucleotide 5' CTAGGCTGGAGCACTGGACACTGTC 3' complementary to position 80-104 in the gene was used as an extension primer. 10  $\mu$ g of total RNA was heated for 10 minutes at 70°C together with  $^{32}$ P 5' end-labeled primer. The mixture was allowed to cool to 30°C over 30 minutes and then kept at 30°C for an additional 30 minutes. To the mixtures were added (final concentration) 50mM Tris-HCl (pH 8.3), 75 mM KCl and 3mM MgCl<sub>2</sub>, 5mM DDT and 250 mM of dATP, dCTP, dGTP, dTTP in 40  $\mu$ l total volume. The reactions were



started by the addition of 200 u Superscript RNase H<sup>-</sup> (GIBCO, Bethesda Research Laboratories) and incubated for 90 minutes at 42°C. Samples were phenol/chloroform-extracted, ethanol-precipitated, resuspended in formamide dye and after heating to 75°C for 3 minutes, separated on a 6% sequencing gel.

## 5 **Transcriptional analysis**

Cultured human skin fibroblasts were grown to confluency on 150 mm tissue culture dishes as described. Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991). Cells were then treated for various time-points with 1 μM RA in pre-warmed and equilibrated Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Nuclei for each  
10 time-point were isolated from four dishes after incubation in lysis buffer containing 0.5% NP-40 as described. Greenberg, M.E. et al., *Nature* 311:433-438 (1984). Nuclear run-on experiments were performed with [ $\alpha$ -<sup>32</sup>P] UTP (DuPont-New England Nuclear, 800 Ci/mmol) as described. Antras, J. et al., *J. Biol. Chem.* 266:1157-1161 (1991). Equal  
15 amounts of radioactivity (0.5-1 x 10<sup>7</sup> cpm) were hybridized to nitrocellulose filters containing 5 μg of each plasmid. After hybridization for 72 hours at 42°C, the filters were washed twice with 2 x SSC at 37°C for 15 minutes and treated for 30 minutes at 37°C in 2 x SSC containing 5 μg/ml RNase A. The filters were then washed twice for 15 minutes  
20 in 2 x SSC, 0.5% SDS at 42°C and once for 30 minutes in 0.5 x SSC, 0.5% SDS at 42°C. A final wash was carried out in 0.1 x SSC, 0.1% SDS for 30 minutes at 55°C. The amount of radioactivity present in each slot was determined using a phosphorimager (Molecular Dynamics) after over-night exposure and autoradiograms were exposed for 4-5 days at -70°C with intensifying screens.

## **RESULTS**

### ***Cloning and characterization of the human CRABP-II gene***

25 Using the human CRABP-II cDNA as a probe, one bacteriophage lambda clone was isolated (λ2.1) from a human placenta genomic library. Restriction mapping and sequencing revealed that this clone contained the entire CRABP-II gene. A restriction map of λ2.1 is shown in Figure 7. The gene is composed of four exons, interrupted by one large and two small introns. The overall size of the gene is approximately 6 kb.

### 30 ***DNA Sequence of the human CRABP-II gene***

To characterize the CRABP-II gene, fragments spanning the entire gene, except for the first intron were subcloned into M13 vectors for sequence analysis. The nucleotide sequence and the deduced amino-acid sequence is presented in Figure 8. The first three exons are small and range in size from 117 to 207 bp, with the fourth exon being the  
35 largest at 466 bp as demonstrated in Figure 8. The exon sequences were found to be



identical to the published cDNA sequence. Aström, A. et al., *J. Biol. Chem.* 266:17662-17666 (1991). All splice junctions contained the expected GT splice donor and AT splice acceptor.

***Analysis of the 5' end of the human CRABP-II gene***

5           The 5' boundary of the first exon was determined by primer extension analysis. Using an oligonucleotide primer complimentary to position 80-104, a predominant reaction product was identified when mRNA from untreated cultured human skin fibroblasts were used, see Figure 9. Comparison of the extension product with sequencing reactions originating from the same primer indicated that the major transcription initiation site should  
10 be assigned to the A residue 137 bp upstream of the ATG. When fibroblasts were treated with 1  $\mu$ M RA for 24 hours an increase in initiation at the A residue could be seen. In addition a second initiation product with the same intensity could be seen at -1.

Sequence analysis of the upstream region revealed a TATA box (TATAAA) at -31 and several potential regulatory elements including two potential AP2 binding sites at -631  
15 and -402, and one potential SP1 site at -89 as demonstrated in Figure 8. Mitchell, P.J. et al., *Cell* 50:847-861 (1987); , Kadonaga, J. T. et al., *Trends Biochem. Sci.* 11:20-23 (1986). In addition two sequences can be found that exactly match a binding site for the early growth response gene *Krox-24* (Egr-1, zif268 or NGFI-A) at -579 and -116. Lemaire, P. et al., *Mol. Cell. Biol.* 10:3456-3467 (1990). In addition a direct repeat spaced by one bp  
20 (AGTTCAgGGTTCA) can be found in the upstream region of the gene at -454, with homology to the retinoic acid responsive element found in the RAR- $\beta_2$  gene. Umesono, K. et al., *Cell* 65:1255-1266.

***Effects of RA on CRABP-II transcription rates and mRNA levels***

To determine whether RA induction of CRABP-II mRNA seen in cultured human skin  
25 fibroblasts was due to increased transcription, nuclear run-on assays were performed using nuclei isolated from cultured human skin fibroblasts treated with RA for various periods of time. As seen in Figure 10, RA caused a rapid transient increase in CRABP-II transcription, peaking at 2 hours and being almost back to control levels at 6 hours. However, there was no change in RAR- $\gamma$  and cyclophilin transcription, two genes known  
30 to be unaffected by RA in skin fibroblasts as demonstrated by Figure 10. Edler, J.T. et al., *J. Invest. Dermatol.* 96:425-433 (1991). Quantitation and normalization to cyclophilin shows that CRABP-II transcription is induced approximately 6-fold after 2 hours of RA treatment. See Figure 11.

This increase in transcription can account for the 6-fold induction of CRABP-II  
35 mRNA after 24 hours of treatment, as seen in Figure 11. Induction of CRABP-II mRNA can

be detected as early as 2 hours after addition of RA to cells, leveling out between 6 to 24 hours of treatment. However, a smaller increase can be seen between 24 and 48 hours. To determine whether RA is required to be present in the medium for maintaining the induced CRABP-II message levels after the transcription has occurred, human skin  
5 fibroblasts were treated with RA for 12 hours, after which the cells were washed two times and replaced with medium without any addition of RA. As can be seen in Figure 11, removal of RA from the medium causes a decline of the CRABP-II message compared to cells treated with RA for the entire experiment.

***Effects of cycloheximide or actinomycin D on the induction of CRABP-II message***

10 Experiments were performed to determine whether the addition of inhibitors of transcription or translation could inhibit the increase of CRABP-II mRNA produced by RA. Cycloheximide (10  $\mu$ g/ml) or actinomycin D (2  $\mu$ g/ml) were added simultaneously with RA (1  $\mu$ M) and CRABP-II levels were determined after 2 hours. As can be seen in Figure 12, cycloheximide down-regulated basal CRABP-II expression and blocked the RA induction.  
15 Cycloheximide had no effect on RAR- $\gamma$  expression or RA induction of RAR- $\beta$  (Figure 12), as previously reported. Nervi, C. et al., *Cell Growth Diff.* 1:535-542 (1990); DeThe, H. et al., *EMBO J.* 8:429-433. Actinomycin D, had no effect on basal levels of CRABP-II message, but completely blocked induction by RA. As controls, RAR- $\gamma$  was found to be down-regulated by actinomycin D, and RA induction of RAR- $\beta$  was completely blocked in  
20 agreement with previous reports. Nervi, C. et al., *Cell Growth Diff.* 1:535-542 (1990); DeThe, H. et al., *EMBO J.* 8:429-433 (1989).

***Effects of cycloheximide on CRABP-II transcription rates***

Since cycloheximide blocked RA induction of CRABP-II mRNA, run-on experiments were performed to see if protein synthesis was required for the induction of transcription.  
25 As can be seen in Figure 13, addition of Cycloheximide at time 0 completely blocked RA induction of CRABP-II gene transcription.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and claims, that various changes, modifications and  
30 variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

All publications and applications cited herein are incorporated by reference.

- 27 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Voorhees, John J.  
Astrom, Anders  
Pattersson, Ulrika  
Tavakkol, Amir

(ii) TITLE OF INVENTION: HUMAN CRABP-I AND CRABP-II

(iii) NUMBER OF SEQUENCES: 6

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Harness, Dickey & Pierce

(B) STREET: PO Box 828

(C) CITY: Bloomfield Hills

(D) STATE: Michigan

(E) COUNTRY: United States of America

(F) ZIP: 48013

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

- 28 -

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Lewak, Anna M.
- (B) REGISTRATION NUMBER: 33,006
- (C) REFERENCE/DOCKET NUMBER: 211500676POB

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (313) 641-1600
- (B) TELEFAX: (313) 641-0270
- (C) TELEX: 287637

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 924 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA to mRNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapien
- (F) TISSUE TYPE: skin
- (G) CELL TYPE: fibroblast

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: HUMAN SKIN FIBROBLAST LAMBDA GT11
- (B) CLONE: LAMBDA F1.1

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(A) NAME/KEY: polyA\_site

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(D) OTHER INFORMATION: /citation= ([1])

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(A) NAME/KEY: 5'UTR

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(A) NAME/KEY: terminator

(B) LOCATION: 513..515

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- 30 -

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Pettersson, Ulrika  
Cromie, Matthew  
Elder, James T.  
Voorhees, John J.
- (B) TITLE: Molecular Cloning of Two Human Cellular  
Retinoic Acid-Proteins (CRABP)
- (C) JOURNAL: J. Biol. Chem.
- (G) DATE: 1991
- (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 924

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Elder, James T.  
Pettersson, Ulrika  
Cromie, Matthew  
Voorhees, John J.
- (B) TITLE: Cloning of CRABPII cDNA from Human Skin:  
Retinoic Acid Induces Expression of CRABPII but  
Not CRABPI in Human Skin in Vivo and in Dermal but  
Not Lung Fibroblasts in Vitro
- (C) JOURNAL: J. Invest. Dermatol.
- (D) VOLUME: 96
- (F) PAGES: 547-547
- (G) DATE: April-1991

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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- 31 -

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 138 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: YES

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: homo sapien

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Pettersson, Ulrika  
Cromie, Matthew  
Elder, James T.  
Voorhees, John J.

- 32 -

(B) TITLE: Molecular Cloning of Two Human Cellular  
Retinoic Acid-Proteins (CRABP)

(C) JOURNAL: J. Biol. Chem.

(G) DATE: 1991

(K) RELEVANT RESIDUES IN SEQ ID NO:2: FROM 1 TO 138

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Elder, James T.  
Pettersson, Ulrika  
Cromie, Matthew  
Voorhees, John J.

(B) TITLE: Cloning of CRABPII cDNA from Human Skin:  
Retinoic Acid Induces Expression of CRABPII but  
Not CRABPI in Human Skin in Vivo and in Dermal but  
Not Lung Fibroblasts in Vitro

(C) JOURNAL: J. Invest. Dermatol.

(D) VOLUME: 96

(F) PAGES: 547-547

(G) DATE: April-1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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- 33 -

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## (2) INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 525 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo Sapien

(F) TISSUE TYPE: skin

(G) CELL TYPE: fibroblast

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: human skin Lambda ZapII

(B) CLONE: lambda s3.1

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(A) NAME/KEY: CDS

(B) LOCATION: 8..418

(D) OTHER INFORMATION: /codon\_start= 8  
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- (A) NAME/KEY: 5'UTR
- (B) LOCATION: 1..7
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## (ix) FEATURE:

- (A) NAME/KEY: 3'UTR
- (B) LOCATION: 419..525
- (D) OTHER INFORMATION: /citation= ([1])

## (ix) FEATURE:

- (A) NAME/KEY: terminator
- (B) LOCATION: 419..421
- (D) OTHER INFORMATION: /citation= ([1])

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Pettersson, Ulrika  
Cromie, Matthew  
Elder, James T.  
Voorhees, John J.
- (B) TITLE: Molecular Cloning of Two Human Cellular  
Retinoic Acid-Proteins (CRABP)
- (C) JOURNAL: J. Biol. Chem.
- (G) DATE: 1991
- (K) RELEVANT RESIDUES IN SEQ ID NO:3: FROM 1 TO 525

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Astrom, Anders  
Tavakkol, Amir  
Elder, James T.  
Pettersson, Ulrika  
Cromie, Matthew  
Voorhees, John J.

- 35 -

(B) TITLE: Cloning of CRABPII cDNA from Human Skin:  
Retinoic Acid Induces Expression of CRABPII but  
Not CRABPI in Human Skin in Vivo and in Dermal but  
Not Lung Fibroblasts in Vitro

(C) JOURNAL: J. Invest. Dermatol.

(D) VOLUME: 96

(F) PAGES: 547-547

(G) DATE: April-1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 137 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

- 36 -

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: homo sapien

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Astrom, Anders  
 Tavakkol, Amir  
 Pettersson, Ulrika  
 Cromie, Matthew  
 Elder, James T.  
 Voorhees, John J.

(B) TITLE: Molecular Cloning of Two Human Cellular  
 Retinoic Acid-Proteins (CRABP)

(C) JOURNAL: J. Biol. Chem.

(G) DATE: 1991

(K) RELEVANT RESIDUES IN SEQ ID NO:4: FROM 1 TO 137

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Astrom, Anders  
 Tavakkol, Amir  
 Elder, James T.  
 Pettersson, Ulrika  
 Cromie, Matthew  
 Voorhees, John J.

(B) TITLE: Cloning of CRABPII cDNA from Human Skin:  
 Retinoic Acid Induces Expression of CRABPII but  
 Not CRABPI in Human Skin in Vivo and in Dermal but  
 Not Lung Fibroblasts in Vitro

(C) JOURNAL: J. Invest. Dermatol.

(D) VOLUME: 96

(F) PAGES: 547-547

(G) DATE: April-1991

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Pro	Asn	Phe	Ala	Gly	Thr	Trp	Lys	Met	Arg	Ser	Ser	Glu	Asn	Phe
1				5				10					15		
Asp	Glu	Leu	Leu	Lys	Ala	Leu	Gly	Val	Asn	Ala	Met	Leu	Arg	Lys	Val
		20					25					30			



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Ala Val Ala Ala Ala Ser Lys Pro His Val Glu Ile Arg Gln Asp Gly  
           35                                  40                                  45  
 Asp Gln Phe Tyr Ile Lys Thr Ser Thr Thr Val Arg Thr Thr Glu Ile  
           50                                  55                                  60  
 Asn Phe Lys Val Gly Glu Gly Phe Glu Glu Glu Thr Val Asp Gly Arg  
           65                                  70                                  75                                  80  
 Lys Cys Arg Ser Leu Ala Thr Trp Glu Asn Glu Asn Lys Ile His Cys  
                                   85                                  90                                  95  
 Thr Gln Thr Leu Leu Glu Gly Asp Gly Pro Lys Thr Tyr Trp Thr Arg  
                                   100                                  105                                  110  
 Glu Leu Ala Asn Asp Glu Leu Ile Leu Thr Phe Gly Ala Asp Asp Val  
                                   115                                  120                                  125  
 Val Cys Thr Arg Ile Tyr Val Arg Glu  
           130                                  135

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1322 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (F) TISSUE TYPE: Placenta

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## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: human placenta genomic library

(B) CLONE: lambda 2.1

## (ix) FEATURE:

(A) NAME/KEY: TATA\_signal

(B) LOCATION: 1008..1013

## (ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 1039..1245

## (ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1246..1322

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Astrom, Anders  
Pettersson, Ulrika  
Voorhees, John J(B) TITLE: Structure of the human cellular retinoic  
acid-binding protein II (CRABP-II) gene: Early  
transcriptional regulation by retinoic acid

(C) JOURNAL: J. Biol. Chem.

(G) DATE: 1992

(K) RELEVANT RESIDUES IN SEQ ID NO:5: FROM 1 TO 1322

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CTGCAGGAAG CCGTGCCCTC CTCCCACCCT CTTTGATCTC CCGTTTCAA GCCGCTCTCC 60
AAGGGAGGGG AGGTCGCTCC TTCCGCCCGT TTTACAGCTC AGGATGGTGA CACCTGAGAC 120
CCTGCTCCGC CTTCTCCCCC GGCACCCATC CTCCGCCTA TCTAGGTGGT GGCAGAGCTC 180
GCCAGGGCTC CGCGCCTCTG TCCCGCCTC CCTCCCTTCC CCCTACTGAG ACCCCTCGGG 240
```

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GTCTCGGGAG TGAAGCGACA GAGAAAGCGT TTTAATAAAG ACCTTGCGTC AAGTGATTGG 300  
CTGTGACCTC TGCCCTCCCA GCCTCGCGCC CTGGGCTCCT GCTTAACCCT TCAATGTCCG 360  
CCCAGCGCAT TAAGGGGAGC GAGTCGCCTG GCGACTACTT CCAGAGTCCC CAGGCATTAC 420  
GTGAGCCCGA AGCAGGGTGG AGGGGTGGGG GGACCGTGCC GCCCCGCCC AGCCTCTCCG 480  
AGTTGTTCCA GCAGGGGGCG CCGTTGCCTC ACTTAGATCC CTAACCCCG GAACCCCGCA 540  
GCTCCCAAGC CCCTCTCTGA GTACGGAGTG GTCCCACTGG ATCCAGTTCA GGGTTCAATG 600  
GAGCTAGGGC CAGCTACGGC TCAAGATCTG GGGTCCGCCT GCGGTGGGGT CGCCAGGTGT 660  
CCGGCACCAA GGAGTTGAAT GCACCGAGTC AGGTTGGGGA TGGGTGGGGA ACAGGCGAGA 720  
CGTGAGGAAC TCGGGTGGGG GACAGCCATA CACGAGCCCT GAGCATCTGC GCCCGCAGCT 780  
AGCTCCCCC GCCTCTGCGG AGAGCGCGAT TCAAGTGCTG GCTTTGCGTC CGCTTCCCCA 840  
TCCACTTACT AGCGCAGGAG AAGGCTATCT CGGTCCCCAG AGAAGCCTGG ACCCACACGC 900  
GGGCTAGATC CAGAGGTTGG TGGCGGGGGC GCAGGGCCCC AGGTGGGGGG GGGCGGAGCG 960  
GGAGGCGGGG CCACTTCAAT CCTGGGCAGG GGC GGTTCCG TACAGGGTAT AAAAGCTGTC 1020  
CGCGCGGGAG CCCAGGCCAG CTTTGGGGTT GTCCCTGGAC TTGTCTTGGT TCCAGAACCT 1080  
GACGACCCGG CGACGGCGAC GTCTCTTTTG ACTAAAAGAC AGTGTCCAGT GCTCCAGCCT 1140  
AGGAGTCTAC GGGGACCGCC TCCGCGCCG CCACCATGCC CAACTTCTCT GGCAACTGGA 1200  
AAATCATCCG ATCGGAAAAC TTCGAGGAAT TGCTCAAAGT GCTGGGTAAG GAAATGTTCG 1260  
AGGGCCCAGG TGGGGCAAGG GGGGGCTCTG GAGTCCTCGA AGTTGGGGAT GAGAAAGACA 1320  
GC 1322

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1717 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

- 40 -

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(F) TISSUE TYPE: Placenta

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: human placenta genomic

(B) CLONE: lambda 2.1

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 178..356

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 357..571

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 572..688

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 699..1152

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 1153..1618

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## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Astrom, Anders  
Pettersson, Ulrika  
Voorhees, John J

(B) TITLE: Structure of the human cellular retinoic-acid  
binding protein (CRABP-II) gene: Early  
transcriptional regulation by retinoic acid

(C) JOURNAL: J. Biol. Chem.

(G) DATE: 1992

(K) RELEVANT RESIDUES IN SEQ ID NO:6: FROM 1 TO 1717

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCTACCGT CTCCTTCAAG GCACTTTCTT AGACACCCGG GCACCAGGCA GATGCACCCC 60  
CCAACACACC CACCCCAAGC AAGTCACAAA TCAGCCTGCT CCAACTGTCT TATGGGGAGG 120  
GTGTGAGAGA GGTGCCCAAA GGCCCCTAAA AGGTGAGCCT CTCCTCTCTC CCCACAGGGG 180  
TGAATGTGAT GCTGAGGAAG ATTGCTGTGG CTGCAGCGTC CAAGCCAGCA GTGGAGATCA 240  
AACAGGAGGG AGACACTTTC TACATCAAAA CCTCCACCAC CGTGCGCACC ACAGAGATTA 300  
ACTTCAAGGT TGGGGAGGAG TTTGAGGAGC AGACTGTGGA TGGGAGGCCC TGTAAGGTGA 360  
GTGCCAGAAG GGGCTCCAGG GTCATGGCGT CATTGCCCTG CCTCTCAACC TGCCATTTTC 420  
CAGGCTAGCA GTTAACTCCT AGCTTCTCTC TGTCCCAGTA GGGAAAATCC CTAGGTAGTG 480  
GTGGGGGCTA GAAAGGGGCT CTCTCCCTTA TCCCTCTCAC TGCATTGCCC CTGCTATGGG 540  
CCCAGCTCAC TTGGCCACCT GTCTCTTGCA GAGCCTGGTG AAATGGGAGA GTGAGAATAA 600  
AATGGTCTGT GAGCAGAAGC TCCTGAAGGG AGAGGGCCCC AAGACCTCGT GGACCAGAGA 660  
ACTGACCAAC GATGGGGAAC TGATCCTGGT AAGTCCTGCC TCCTCCCCAC TAATAGCAAA 720  
CCCAGTGCTA CCTTCCAAGA TTCTCTGGGA GACCCCAGGG TGCAGGAGAC TCAAGAACAA 780  
CCATGGCTGG ACTCCGCACC CTGCTGATGG GACTGCTTGA ACAGAACTAA GGTGTCCCTA 840  
TCCCATACAG TGCCCTGTGT GAATTAGAAA TGGTGTTCCT TTTATGCAAG CAAAGGGCAT 900  
GTA CTGAGGG ATCCCAGCAG TTCTTCAGGG AGATCTTCCT GGCTTGAGGA GGAGGACGGG 960  
CCCCAGGGCT CTATTGCTAT CCTCCCTCCA TTGATGCCTG GGCATTCTGG GACCAGCTCC 1020  
TGCCTGTTGG TCTTGAGCCA AGAAGCAGGT TTGGACCTGG AGGCCAAGCA GAGTACCTCC 1080

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ATTCAACCCT CCTCTCCAAA GCCACAGGAC CCCAGGGGCC TCTCAGGCTA ACAACTACTT 1140  
CTGTCCTTCC AGACCATGAC GGC GGATGAC GTTGTGTGCA CCAGGGTCTA CGTCCGAGAG 1200  
TGAGTGGCCA CAGGTAGAAC CGCGGCCGAA GCCCACCCTT GGCCATGCTC ACCGCCCTGC 1260  
TTCCTGCCC CCTCCGTCCC ACCCCCTCCT TCTAGGATAG CGCTCCCCTT ACCCCAGTCA 1320  
CTTCTGGGGG TCACTGGGAT GCCTCTTGCA GGGTCTTGCT TTCTTTGACC TCTTCTCTCC 1380  
TCCCCTACAC CAACAAAGAG GAATGGCTGC AAGAGCCCAG ATCACCCTATT CCGGGTTCAC 1440  
TCCCCGCCTC CCAAGTCAG CAGTCCTAGC CCCAAACCAG CCCAGAGCAG GGTCTCTCTA 1500  
AAGGGGACTT GAGGGCCTGA GCAGGAAAGA CTGGCCCTCT AGCTTCTACC CTTTGTCCCT 1560  
GTAGCCTATA CAGTTTAGAA TATTTATTTG TTAATTTTAT TAAAATGCTT TAAAAAATA 1620  
AAACCTGTCT CTGGCTCATT GGCAGGTAG ATAAGTCACC TGAGTTCAAC CTTGCCTCTG 1680  
AAATGTAGTA TGGGAAAGAC TTGTGTTTCT GCAGCAT 1717



**WHAT IS CLAIMED IS:**

1. The nucleic acid sequence substantially represented by SEQ ID NO. 6, wherein the sequence is isolated from a human cell or is not of human *in vivo* origin.
2. A nucleic acid sequence substantially complementary to SEQ ID NO. 6,  
5 wherein the complementary sequence is isolated from a human cell or is not of human *in vivo* origin.
3. The nucleic acid sequence of Claim 1, wherein the residues represented by T are uracil residues.
4. The nucleic acid sequence of Claim 2, wherein the residues represented by  
10 T are uracil residues.
5. The nucleic acid sequence substantially represented by a sequence selected from the group consisting essentially of SEQ ID NOS. 1, 5 and 6, wherein the sequence is isolated from a human cell or is not of human *in vivo* origin.
6. A nucleic acid sequence substantially complementary to a sequence  
15 selected from the group consisting essentially of SEQ ID NOS. 1, 5 and 6, wherein the complementary sequence is isolated from a human cell or is not of human *in vivo* origin.
7. The nucleic acid sequence of Claim 5, wherein the residues represented by T are uracil residues.
8. The nucleic acid sequence of Claim 6, wherein the residues represented by  
20 T are uracil residues.
9. The amino acid sequence substantially similar to SEQ ID NO. 2, wherein the amino acid sequence is isolated from a human cell or is not of human *in vivo* origin.
10. An antibody or binding fragment thereof raised to at least a portion of the amino acid sequence substantially similar to SEQ ID NO. 2.

11. A nucleic acid probe comprising at least a segment of the nucleic acid sequence represented by a sequence selected from the group consisting essentially of SEQ ID NOS. 1, 5 and 6, wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto, and wherein the residues represented by T are selected  
5 from the group consisting of thymine and uracil.

12. The complementary sequence of the probe of Claim 11.

13. A method of screening a sample for the expression of human CRABP-II comprising the steps of:

- 10
- a) providing a nucleic acid probe complementary to a portion of the nucleic acid sequence represented by SEQ ID NO. 1, wherein the residues represented by T are selected from the group consisting of thymine or uracil and the probe is of a length sufficient to hybridize with a complementary sequence thereto;
  - 15 b) contacting the sample under conditions favorable for hybridization of the probe to complementary sequences of nucleic acid in the sample;
  - c) providing means for detecting hybridization; and
  - d) detecting hybridization.

14. The method of Claim 13, further comprising the steps of:

- 20
- e) providing means for quantifying hybridization of the probe to complementary sequences; and
  - f) employing the means for quantifying hybridization.

15. A method of screening a sample for the presence of CRABP-II comprising the steps of:

- 25
- a) providing antibody or binding fragment thereof to CRABP-II;
  - b) contacting the sample under conditions favorable for binding of the antibody or binding fragment to CRABP-II in the sample; and
  - c) providing and employing means for detecting binding.

16. The method of Claim 15, further comprising the steps of:  
d) providing and employing means for quantifying binding of the antibody or fragment to CRABP-II.
17. A nucleic acid sequence substantially similar to SEQ ID NO. 3, wherein the  
5 sequence is isolated from a human cell or is not of human *in vivo* origin.
18. A nucleic acid sequence substantially complementary to the sequence represented by SEQ ID NO. 3, wherein the sequence is isolated from a human cell or is not of human *in vivo* origin.
19. The nucleic acid sequence of Claim 18, wherein the residues represented  
10 by T are uracil residues.
20. The nucleic acid sequence of Claim 19, wherein the residues represented by T are uracil residues.
21. A nucleic acid probe comprising at least a segment of a nucleic acid sequence selected from the group consisting of the sequence substantially represented  
15 by SEQ ID NO. 3 and the sequence substantially complementary thereto, wherein the residues represented by T are selected from the group consisting of thymine and uracil, and wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto.
22. An amino acid sequence substantially represented by SEQ ID NO. 4,  
20 wherein residue 86 is alanine, and wherein the sequence is isolated from a human cell or is not of human *in vivo* origin.
23. An antibody or binding fragment thereto raised to at least a portion of the amino acid sequence of Claim 22.
24. A CRABP-II vector comprising a plasmid or virus including the sequence  
25 substantially represented by SEQ ID NO. 1 or the sequence substantially complementary thereto or both.

25. A CRABP-I vector comprising a plasmid or virus including the sequence substantially represented by SEQ ID NO. 3 or the sequence substantially complementary thereto or both.

26. A recombinant expression construct comprising a nucleic acid sequence  
5 coding for a retinoid-binding protein, the sequence substantially represented by SEQ ID NO. 1 and operatively contained in a cell.

27. The construct of Claim 26, further comprising a reporter element comprising a retinoid responsive element and a reporter gene operatively linked thereto.

28. The construct of Claim 26, further comprising a nucleic acid sequence for  
10 at least the DNA binding domain of a second retinoid-binding protein.

29. The construct of Claim 26, wherein the second retinoid-binding protein is an RAR or an RXR.

30. A recombinant expression construct comprising a cell containing the sequence substantially represented by SEQ ID NO. 3 and further including a reporter  
15 element containing a retinoid responsive element operatively linked to a reporter gene.

31. A reporter assay system comprising a CV-1 cell cotransfected with an expression vector of CRABP-II and an expression vector for an additional retinoid-binding protein, a reporter element in operative configuration with said expression vectors, the reporter element comprising a reporter gene functionally linked to a retinoid responsive  
20 element.

32. The reporter assay of Claim 31, wherein the retinoid-binding protein is an RAR.

33. The reporter assay of Claim 31, wherein the retinoid-binding protein is an RXR.

34. A method for assaying binding of a putative ligand to a receptor protein comprising the steps of:

- a) providing a cell for transfection;
- b) providing a vector comprising a plasmid or virus including a nucleic acid sequence selected from the group consisting of the sequence substantially represented by SEQ ID NO.1, the sequence substantially represented by SEQ ID NO. 2, and the sequences substantially complementary thereto;
- c) transfecting the cell with the vector of step b);
- d) providing a retinoid responsive element;
- e) transfecting the cell with the retinoid-responsive element;
- f) exposing the cell transfected with the vector and the responsive element to the putative ligand under conditions favorable for binding to occur; and
- g) providing and employing means for detecting binding.

35. The method of Claim 34, wherein means for detecting comprises a reporter gene functionally linked to the retinoid responsive element and employing means for detecting comprises assaying for the presence of reporter gene product.

36. A nucleic acid probe comprising at least a segment of the nucleic acid sequence represented by SEQ ID NO. 3, wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto, and wherein the residues represented by T are selected from the group consisting of thymine and uracil.

37. The complementary sequence of the probe of Claim 36.

38. The cellular retinoic acid binding protein encoded by SEQ ID NO. 6.

## AMENDED CLAIMS

[received by the International Bureau on 30 September 1993 (30.09.93);  
original claims 1-8, 11-14, 17-21, 36 and 37 amended;  
other claims unchanged (5 pages)]

1. The nucleic acid substantially represented by SEQ ID NO. 6, wherein the nucleic acid encodes human CRABP-II and is isolated from a human cell or is not of human *in vivo* origin.
- 5 2. A nucleic acid having a sequence substantially complementary to SEQ ID NO. 6, which encodes human CRABP-II wherein the nucleic acid is isolated from a human cell or is not of human *in vivo* origin.
3. The nucleic acid of Claim 1, wherein the residues represented by T are uracil residues.
- 10 4. The nucleic acid of Claim 2, wherein the residues represented by T are uracil residues.
5. The nucleic acid substantially represented by a sequence selected from the group consisting of SEQ ID NOS. 1, 5 and 6, wherein the nucleic acid encodes human CRABP-II, I and II, respectively, and is isolated from a human cell or is not of human *in vivo*  
15 origin.
6. A nucleic acid substantially complementary to a nucleic acid encoding human CRABP-II or I and having a sequence selected from the group consisting of SEQ ID NOS. 1, 5 and 6, wherein the complementary nucleic acid is isolated from a human cell or is not of human *in vivo* origin.
- 20 7. The nucleic acid of Claim 5, wherein the residues represented by T are uracil residues.
8. The nucleic acid of Claim 6, wherein the residues represented by T are uracil residues.
9. The amino acid sequence substantially similar to SEQ ID NO. 2, wherein the  
25 amino acid sequence is isolated from a human cell or is not of human *in vivo* origin.



10. An antibody or binding fragment thereof raised to at least a portion of the amino acid sequence substantially similar to SEQ ID NO. 2.

11. A probe comprising a nucleic acid having a sequence comprising at least a segment of the nucleic acid sequence represented by a sequence selected from the group consisting of SEQ ID NOS. 1, 5 and 6, which encode human CRABP-II, I and II, respectively, wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto under stringent conditions, and wherein the residues represented by T are selected from the group consisting of thymine and uracil.

12. A nucleic acid having a sequence complementary to the probe of Claim 11.

13. A method of screening a sample for the expression of human CRABP-II comprising the steps of:

- a) providing a nucleic acid probe complementary to a portion of the nucleic acid sequence represented by SEQ ID NO. 1, wherein the residues represented by T are selected from the group consisting of thymine or uracil and the probe is of a length sufficient to hybridize under stringent conditions with a complementary sequence thereto;
- b) contacting the sample to complementary sequences of nucleic acid in the sample under conditions favorable for hybridization of the probe;
- c) providing means for detecting hybridization; and
- d) detecting hybridization, wherein expression of human CRABP-II is detected.

14. The method of Claim 13, further comprising the steps of:

- e) providing means for quantifying hybridization of the probe to complementary sequences; and
- f) employing the means for quantifying hybridization, wherein expression of human CRABP-II is detected.

15. A method of screening a sample for the presence of CRABP-II comprising the steps of:
- a) providing antibody or binding fragment thereof to CRABP-II;
  - b) contacting the sample under conditions favorable for binding of the antibody or binding fragment to CRABP-II in the sample; and
  - c) providing and employing means for detecting binding.
16. The method of Claim 15, further comprising the steps of:
- d) providing and employing means for quantifying binding of the antibody or fragment to CRABP-II.
17. A nucleic acid having a sequence substantially represented by SEQ ID NO. 3, wherein the nucleic acid encodes human CRABP-I and is isolated from a human cell or is not of human *in vivo* origin.
18. A nucleic acid substantially complementary to the nucleic acid represented by SEQ ID NO. 3 which encodes human CRABP-I, wherein the nucleic acid is isolated from a human cell or is not of human *in vivo* origin.
19. The nucleic acid of Claim 18, wherein the residues represented by T are uracil residues.
20. The nucleic acid of Claim 19, wherein the residues represented by T are uracil residues.
21. A nucleic acid probe comprising at least a segment of a nucleic acid selected from the group consisting of the nucleic acids having a sequence substantially represented by SEQ ID NO. 3 which encodes human CRABP-I and the sequence substantially complementary thereto, wherein the residues represented by T are selected from the group consisting of thymine and uracil, and wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto under stringent conditions.
22. An amino acid sequence substantially represented by SEQ ID NO. 4, wherein residue 86 is alanine, and wherein the sequence is isolated from a human cell or is not of human *in vivo* origin.

23. An antibody or binding fragment thereto raised to at least a portion of the amino acid sequence of Claim 22.

24. A CRABP-II vector comprising a plasmid or virus including the sequence substantially represented by SEQ ID NO. 1 or the sequence substantially complementary thereto or both.

25. A CRABP-I vector comprising a plasmid or virus including the sequence substantially represented by SEQ ID NO. 3 or the sequence substantially complementary thereto or both.

26. A recombinant expression construct comprising a nucleic acid sequence coding for a retinoid-binding protein, the sequence substantially represented by SEQ ID NO. 1 and operatively contained in a cell.

27. The construct of Claim 26, further comprising a reporter element comprising a retinoid responsive element and a reporter gene operatively linked thereto.

28. The construct of Claim 26, further comprising a nucleic acid sequence for at least the DNA binding domain of a second retinoid-binding protein.

29. The construct of Claim 26, wherein the second retinoid-binding protein is an RAR or an RXR.

30. A recombinant expression construct comprising a cell containing the sequence substantially represented by SEQ ID NO. 3 and further including a reporter element containing a retinoid responsive element operatively linked to a reporter gene.

31. A reporter assay system comprising a CV-1 cell cotransfected with an expression vector of CRABP-II and an expression vector for an additional retinoid-binding protein, a reporter element in operative configuration with said expression vectors, the reporter element comprising a reporter gene functionally linked to a retinoid responsive element.

32. The reporter assay of Claim 31, wherein the retinoid-binding protein is an RAR.

33. The reporter assay of Claim 31, wherein the retinoid-binding protein is an RXR.

5 34. A method for assaying binding of a putative ligand to a receptor protein comprising the steps of:

- 10 a) providing a cell for transfection;
- b) providing a vector comprising a plasmid or virus including a nucleic acid sequence selected from the group consisting of the sequence substantially represented by SEQ ID NO.1, the sequence substantially represented by SEQ ID NO. 2, and the sequences substantially complementary thereto;
- c) transfecting the cell with the vector of step b);
- d) providing a retinoid responsive element;
- 15 e) transfecting the cell with the retinoid-responsive element;
- f) exposing the cell transfected with the vector and the responsive element to the putative ligand under conditions favorable for binding to occur; and
- g) providing and employing means for detecting binding.

20 35. The method of Claim 34, wherein means for detecting comprises a reporter gene functionally linked to the retinoid responsive element and employing means for detecting comprises assaying for the presence of reporter gene product.

25 36. A nucleic acid probe comprising at least a segment of the nucleic acid sequence represented by SEQ ID NO. 3 which encodes human CRABP-I, wherein the segment is of a length sufficient to hybridize to a complementary sequence thereto under stringent conditions, and wherein the residues represented by T are selected from the group consisting of thymine and uracil.

37. A nucleic acid having a sequence complementary to the probe of Claim 36.

38. The cellular retinoic acid binding protein encoded by SEQ ID NO. 6.

Forward: gaattctagaCTGCCACCATGCCCAACTTCGCCCGGTACCTGGAAGATG

Reverse: cactggatccAAGCTGGCCACCTTTCACCTCCCGGACATAAATCCTCGTGCA

T C

T A A

FIG. 1A.

Forward primer: 5'-CAT CGG ATC CCA ACT GGA AGA TCA TCC GA-3'

Reverse primer: 5'-CAT CGG ATC CCA ACG TCA TCT GCT GTC ATT-3'

T T A

T A G A C G

A A C G

G

FIG. 1B.

mCRABP-I	1	----	A-T--MRS	----	D----	A----	A----	V-----	H----	R-D--Q	50
hCRABP-I	1	----	A-T--MRS	----	D----	A----	A----	V-----	H----	R-D--Q	50
hCRABP-II	1	MPN	FSGNWKI	IRSEN	FEELL	KVLGV	NVMLR	KIAVA	AAASK	PAVEIKQEGDT	50
mCRABP-II	1	----	-----	-----	M--A--	M--M--	-----	-----	-----	N--	50

mCRABP-I	51	-----	-----	-----	G--E--	K-R--PT--	N----	IH-T-T-	100	
hCRABP-I	51	-----	-----	-----	G--E--	K-R--AT--	N----	IH-T-T-	100	
hCRABP-II	51	FYIK	STTVRT	TEINF	KVGEE	FEETVD	GRPCK	SLVKE	SENKMVCEQKL	100
mCRABP-II	51	-----	-----	I-----	-----	-----	G-----	-----	R--	100

mCRABP-I	101	-E-D-	-----	Y-----	A--*	-----	FG-----	I-----	137
hCRABP-I	101	-E-D-	-----	Y-----	A--*	-----	FG-----	I-----	137
hCRABP-II	101	LKGE	GPKTSW	TRELT	NDGEL	ILTM	ADDV	CTRVVRE	138
mCRABP-II	101	-----	-----	S-----	-----	-----	-----	-----	138

FIG. 2A.



hCRABP-I	1	M	P	N	F	A	G	T	W	K	M	R	S	S	E	N	F	D	E	L	L	K	A	L	G	V	N
mCRABP-I	M	P	N	F	A	G	T	W	K	M	R	S	S	E	N	F	D	E	L	L	K	A	L	G	V	N	
rCRABP-I		P	N	F	A	G	T	W	K	M	R	S	S	E	N	F	D	E	L	L	K	A	L	G	V	N	
cCRABP-I		P	N	F	A	G	T	W	K	M	R	S	S	E	N	F	D	E	L	L	K	A	L	G	V	N	

hCRABP-II	1	M	P	N	F	S	G	N	W	K	I	I	R	S	E	N	F	E	E	L	L	K	V	L	G	V	N
mCRABP-II	M	P	N	F	S	G	N	W	K	I	I	R	S	E	N	F	E	E	M	L	K	A	L	G	V	N	
rCRABP-II		T	N	F	L	E	N	W	K	I	I	X	S	E	N	F	E	E	M	L	K	A	L	G	V	N	
cCRABP-II		P	N	F	S	G	N	W	K	M	R	S	S	E	N	F	E	E	L	L	K	A	L	G	V	N	

FIG. 2B.

a b c a b c a b c

28S -

18S -

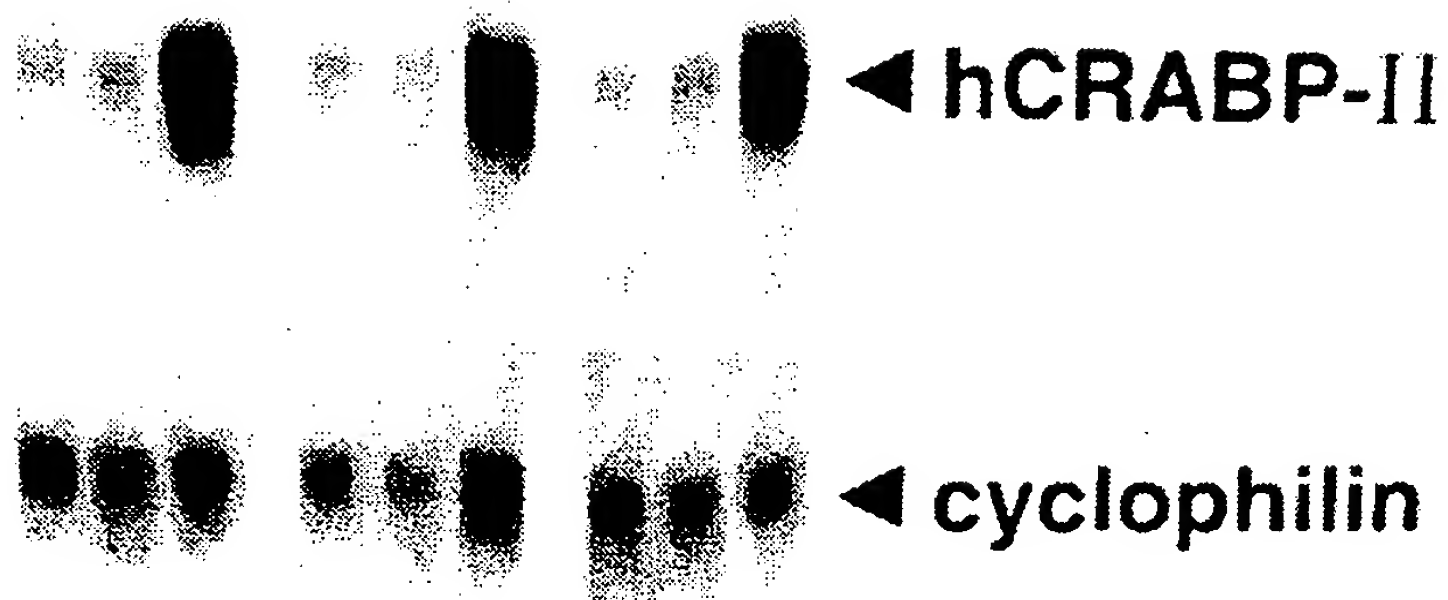
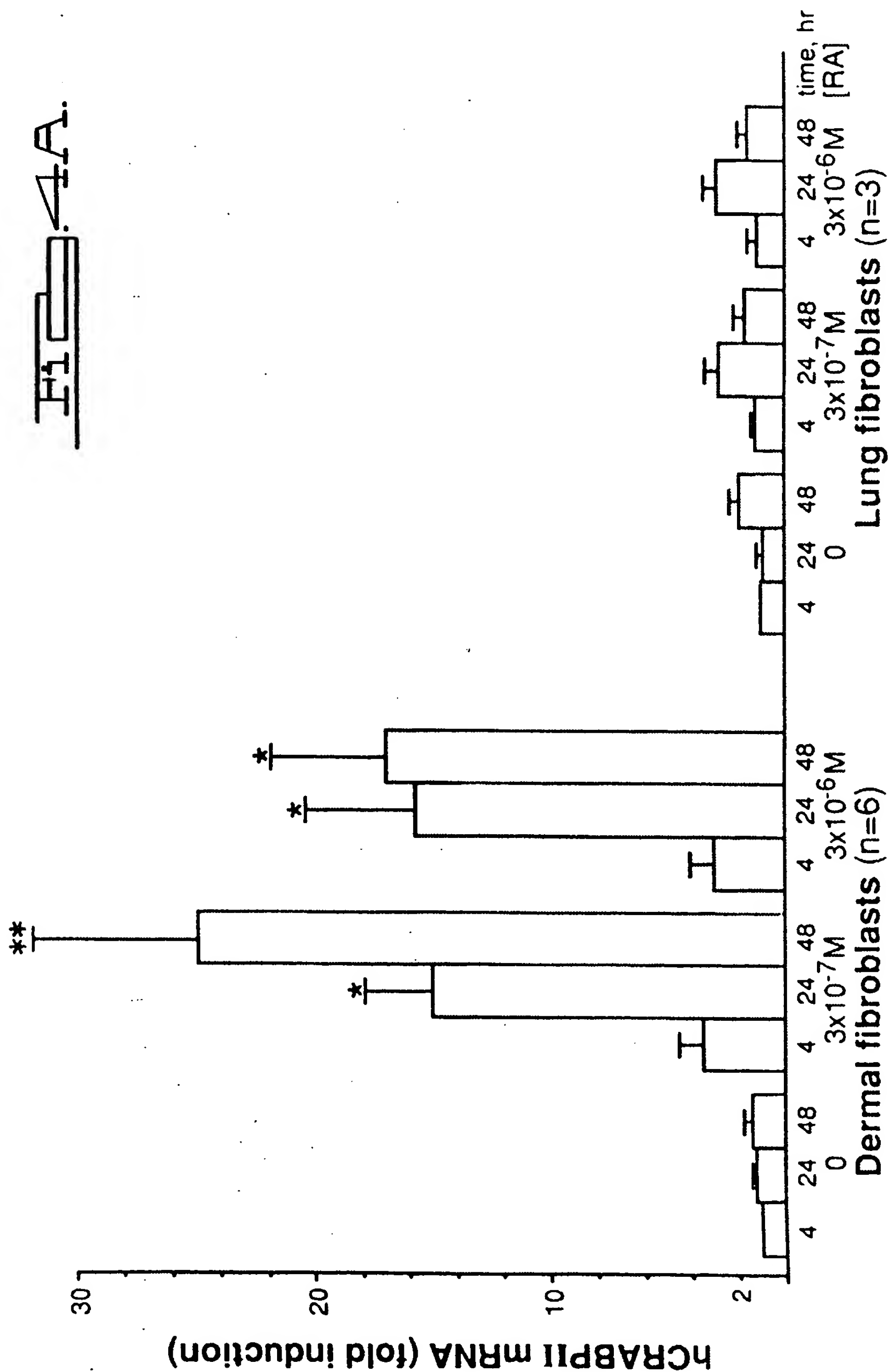
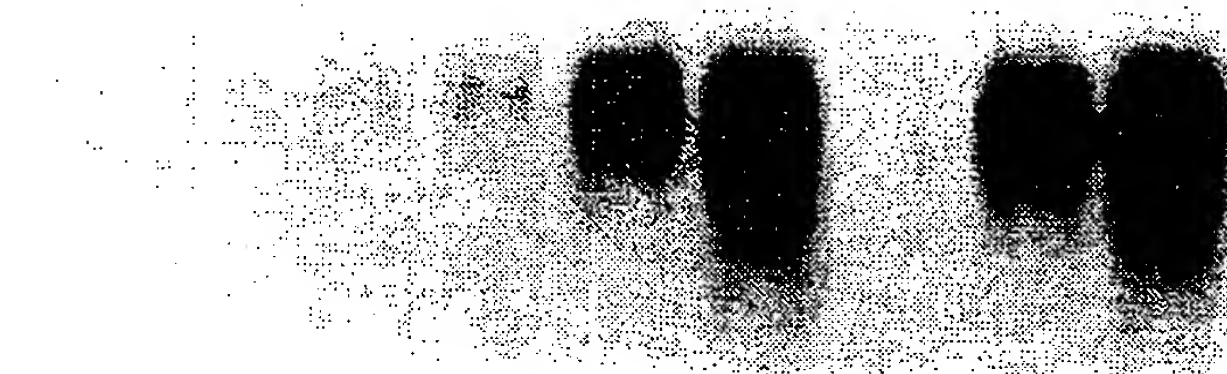


Fig. 3.



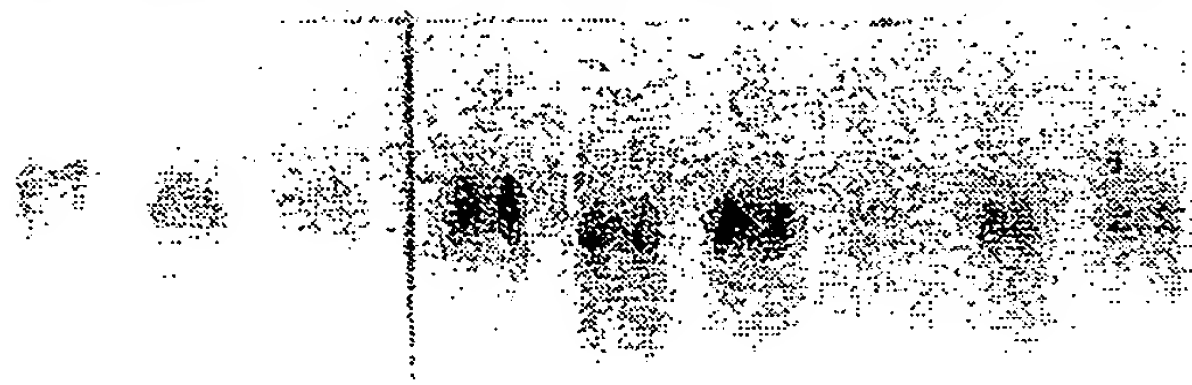
0			0.3			3			[RA], $\mu\text{M}$
4	24	48	4	24	48	4	24	48	time, hr



**dermal FB**

Fi 4B.

0			0.3			3			[RA], $\mu$ M time, hr
4	24	48	4	24	48	4	24	48	



lung FB

Fi 1.4 C.

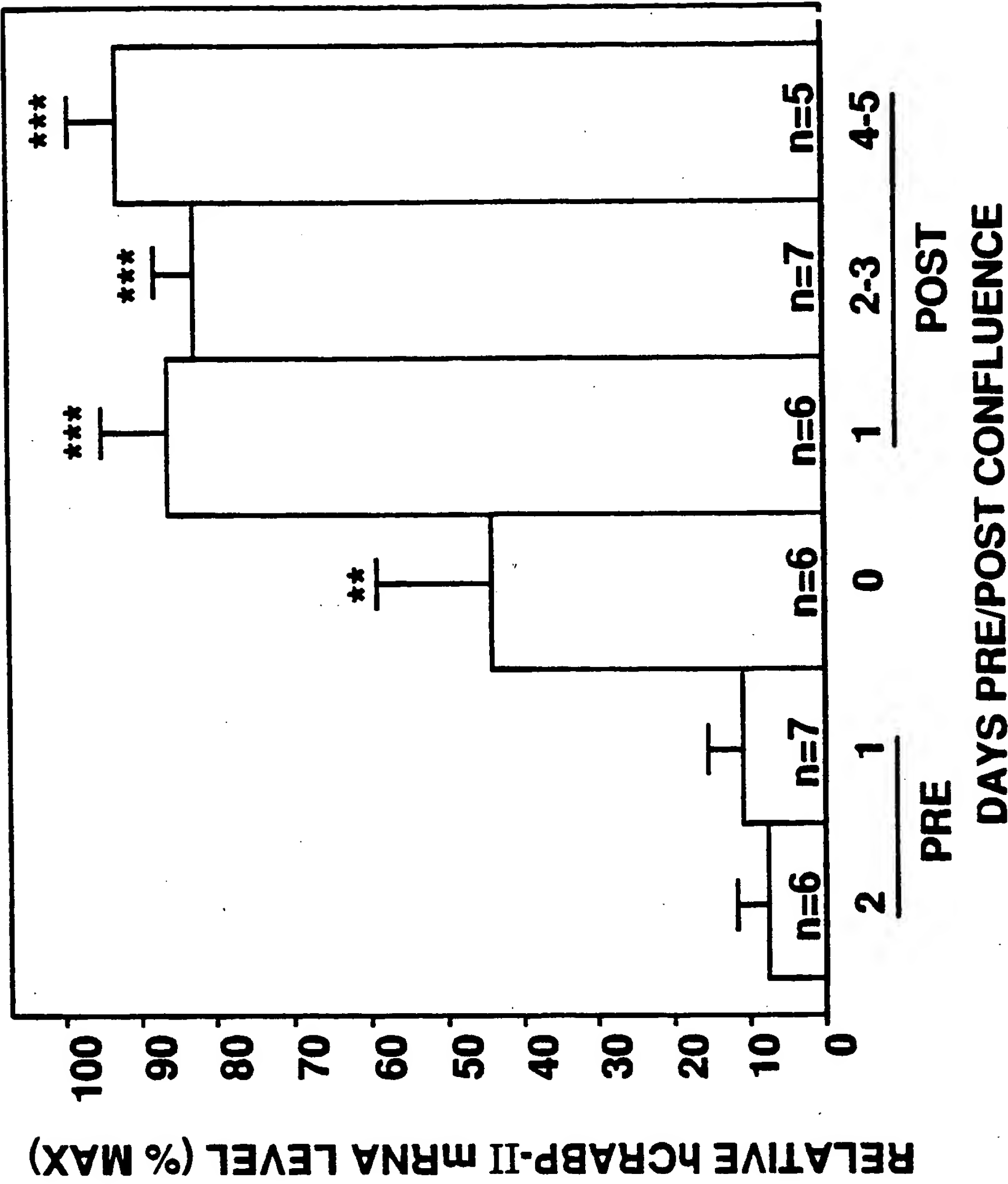


Figure 5A

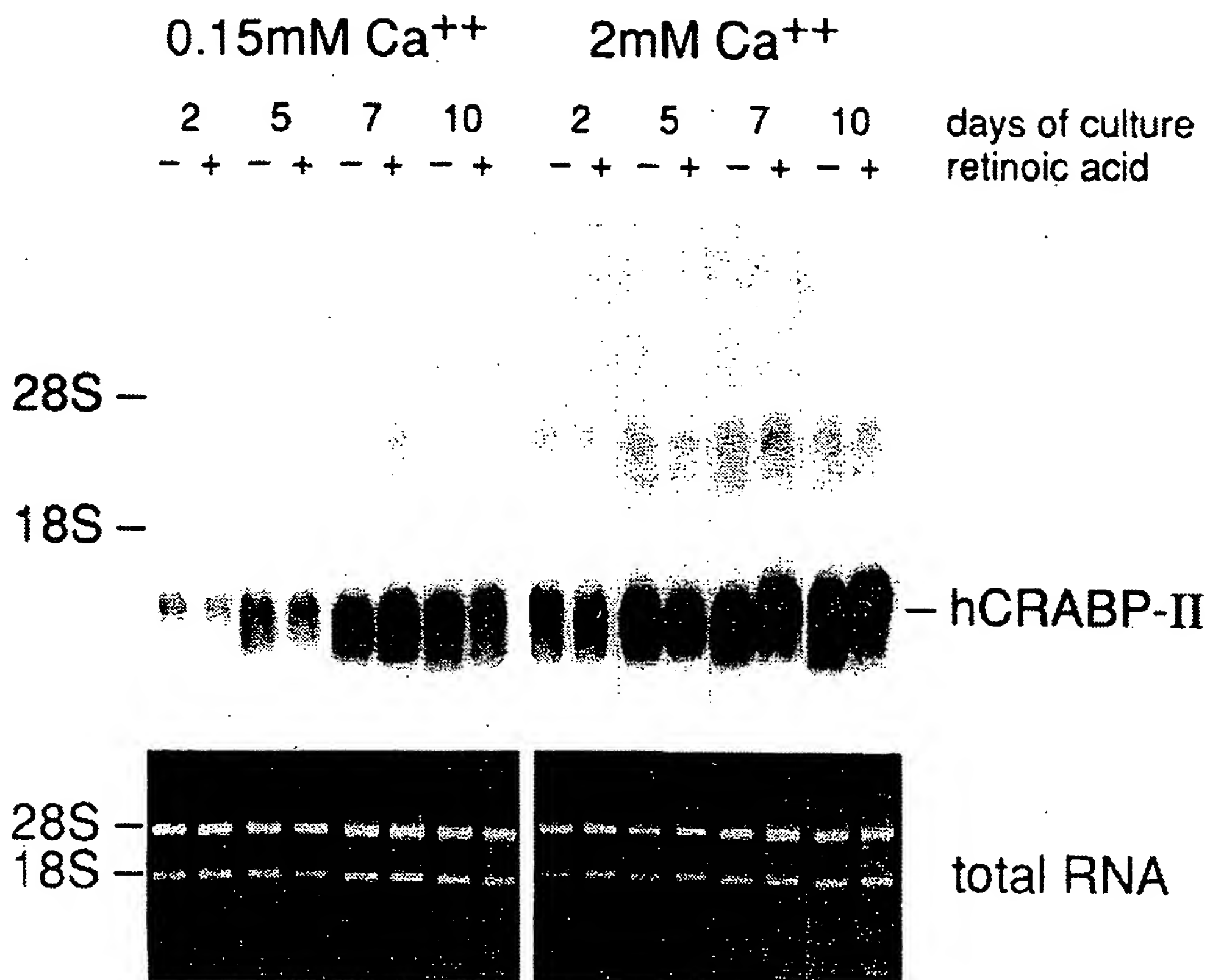


Fig. 5B.



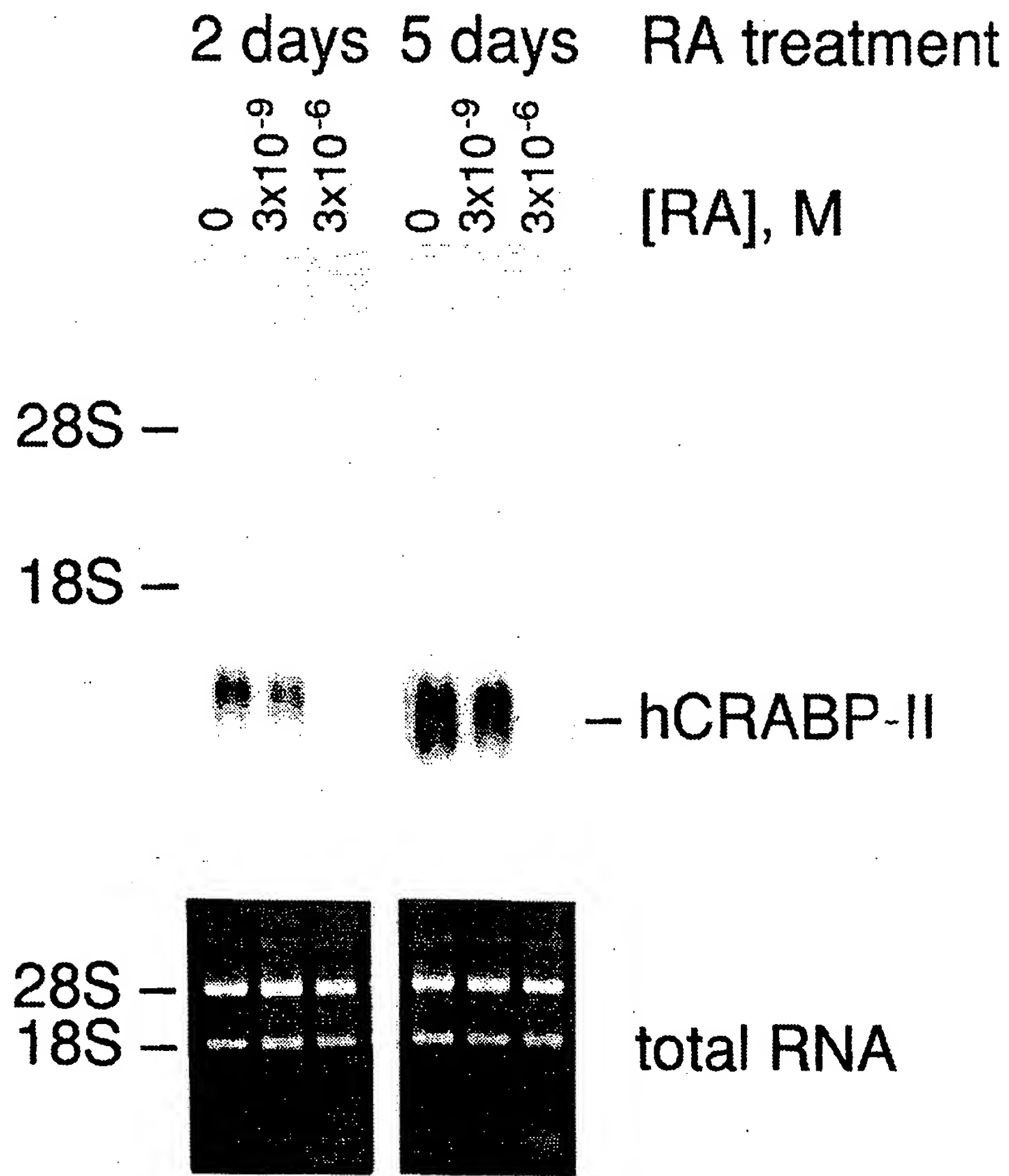


Fig. 5C.

Figure 6

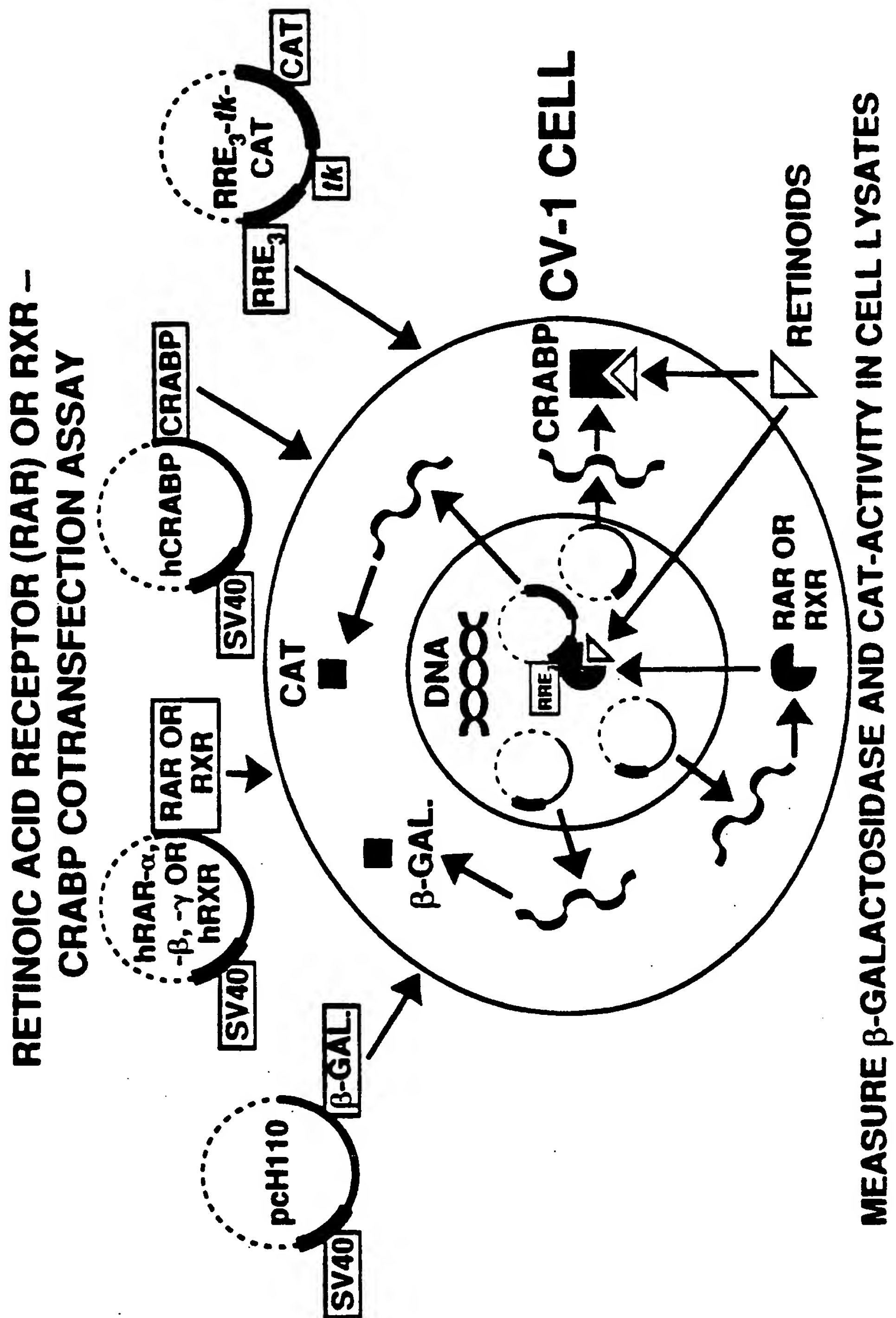
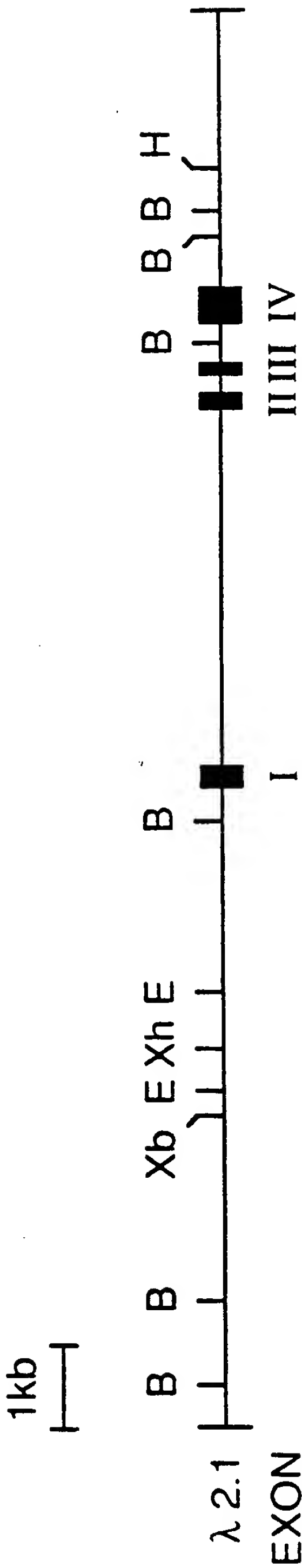
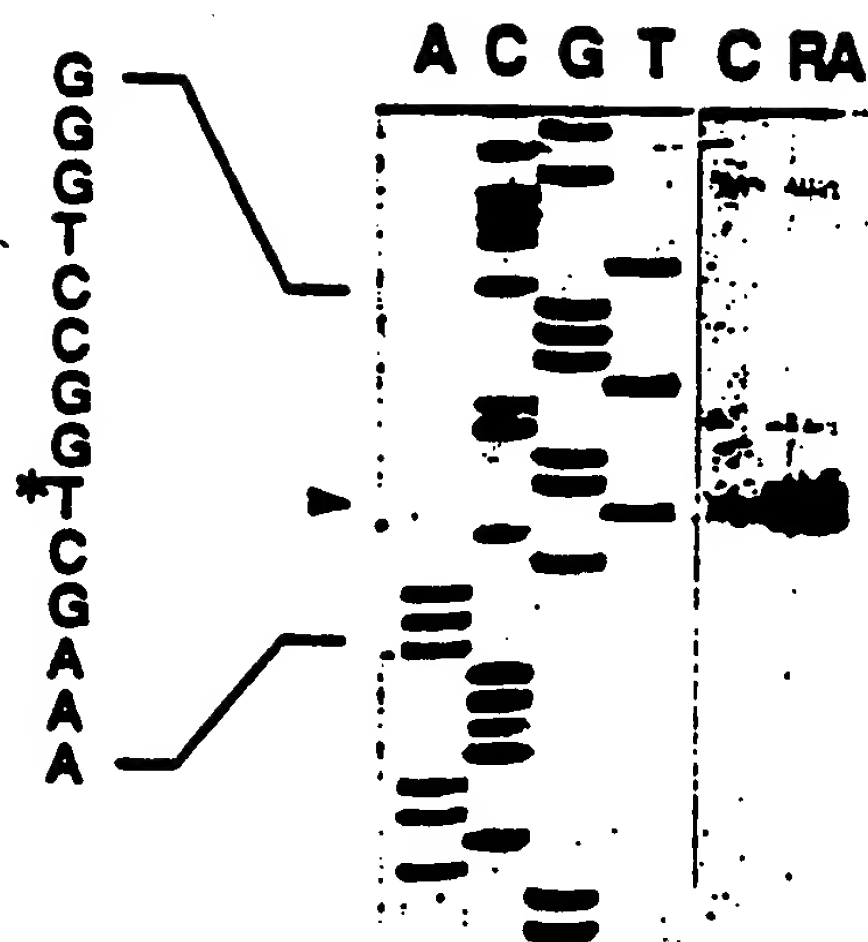


Figure 7



[illegible]

FIGURE 9



10 hr  
10 hr

FIGURE 10

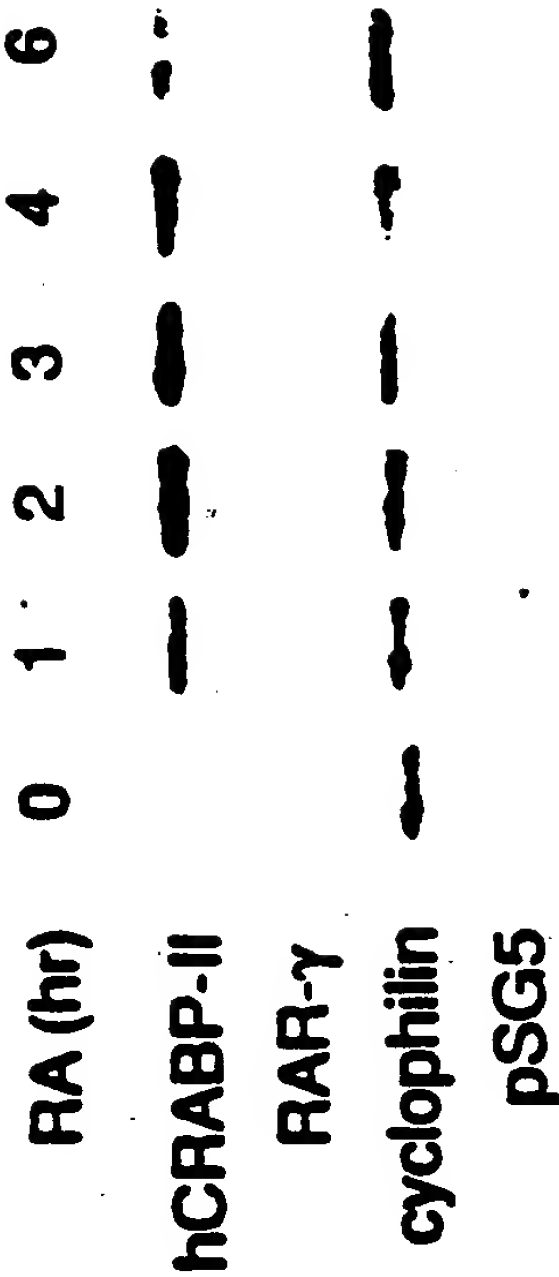


FIGURE 11

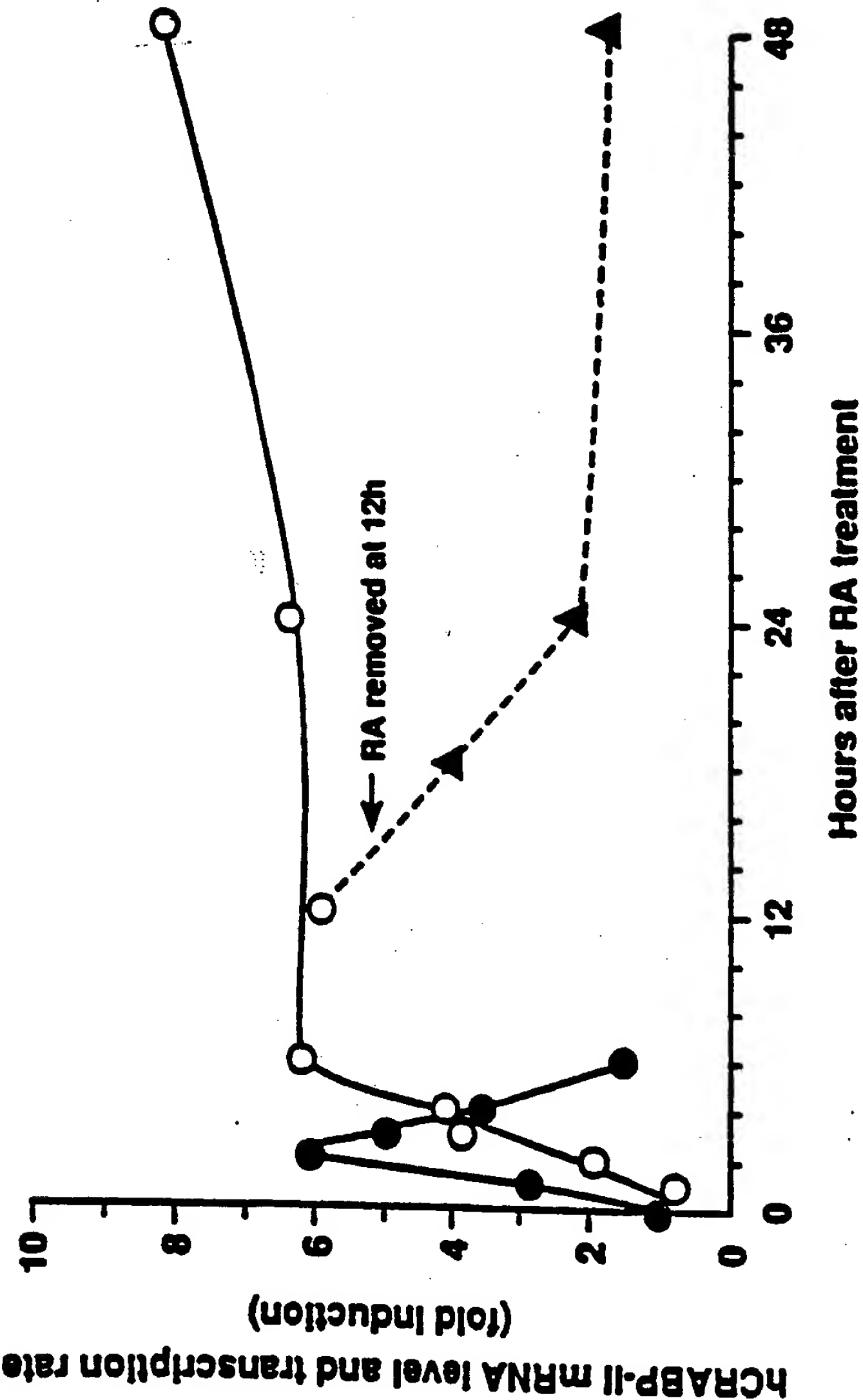




FIGURE 12

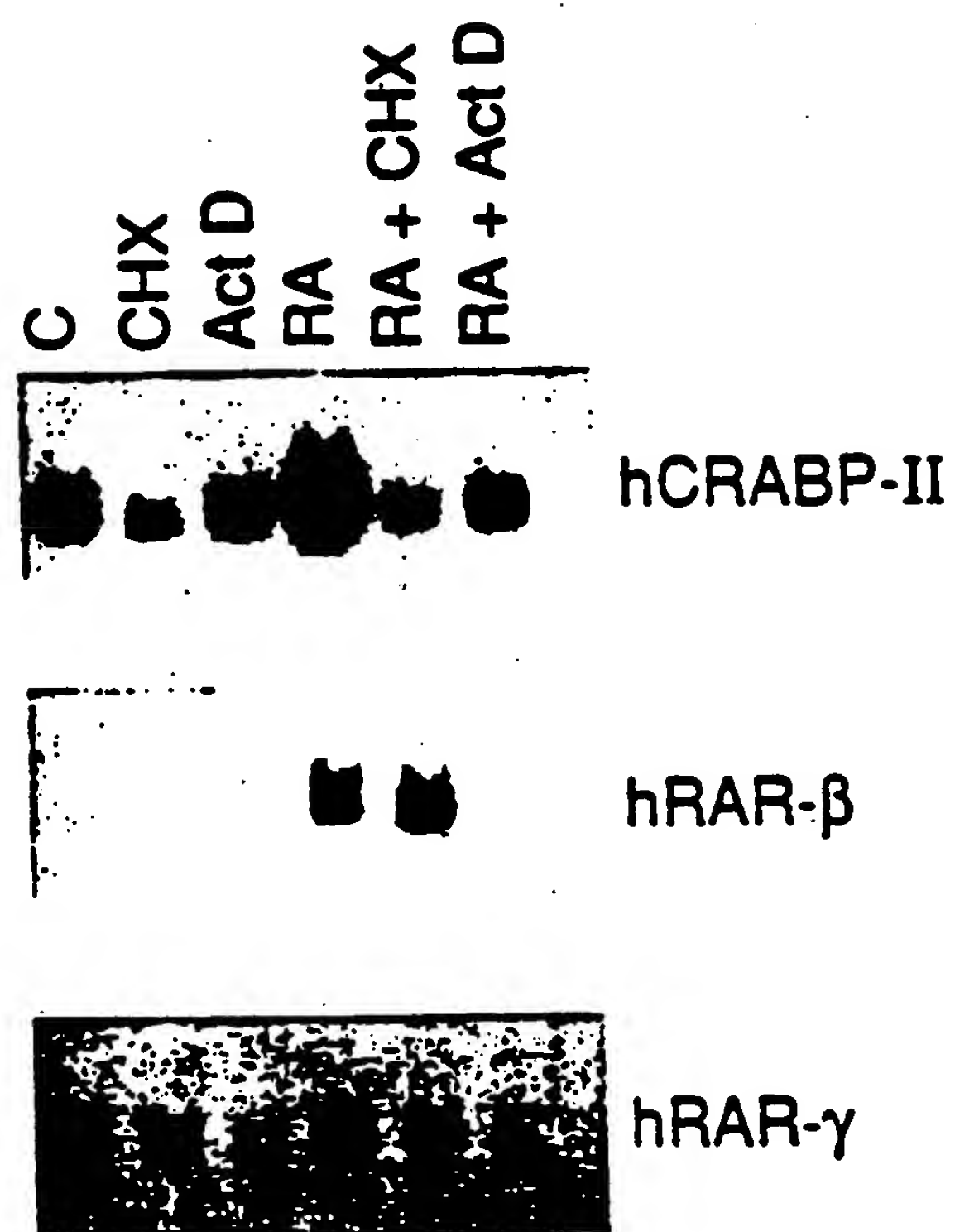
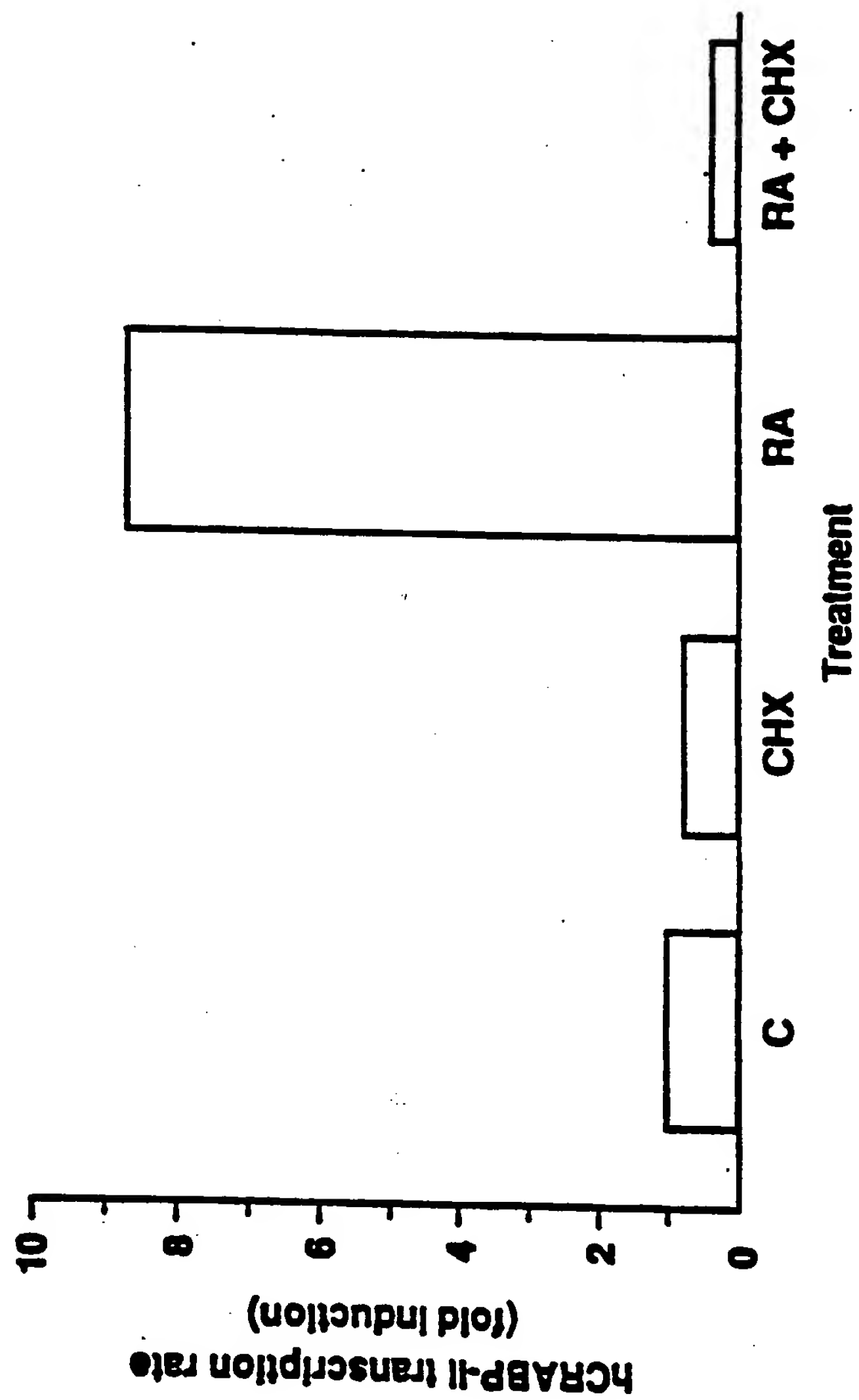


FIGURE 13



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/03936

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C07H 21/04; C12Q 1/68; C07K 15/26; C12N 15/12, 15/63; G01N 33/53  
US CL : 536/23.5, 24.31; 435/6, 7.1, 320.1; 530/ 350, 387.9

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 24.31; 435/6, 7.1, 320.1; 530/ 350, 387.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA ON-LINE, MEDLINE, BIOSIS, GENBANK, UEMBL, SWISSPROT, PIR

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 87, issued August 1990, V. Giguere et al., "Molecular cloning of cDNA encoding a second cellular retinoic acid-binding protein", pages 6233-6237, see entire document.	<u>1-8, 11-12, 17-21, 36-37</u> 9-10, 13-16, 22-35, 38
<u>X</u> Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 173, issued 1988, M.H.L. Nilsson et al., "Isolation and characterization of a cDNA clone corresponding to bovine cellular retinoic-acid-binding protein and chromosomal localization of the corresponding human gene", pages 45-51, see entire document.	<u>1-8, 11-12, 17-21, 24, 25, 36, 37</u> 9-10, 13-16, 22-23, 26-35, 38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	Z	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 JULY 1993	Date of mailing of the international search report 03 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer LISA BENNETT ARTHUR Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/03936

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 84, issued August 1987, H.E Shubeita et al., "Molecular cloning and analysis of functional cDNA and genomic clones encoding bovine cellular retinoic acid-binding protein", pages 5645-5649, see entire document.	1-38
Y	DNA AND CELL BIOLOGY, Volume 9, No. 7, issued 1990, L.-N. Wei et al., "Molecular cloning and transcriptional mapping of the mouse cellular retinoic acid-binding protein gene", pages 471-478, see entire document.	1-38
Y	CANCER RESEARCH, Volume 49, issued 15 March 1989, C.M. Stoner et al., "Mouse cellular retinoic acid binding protein: cloning, complementary DNA sequence and messenger RNA expression during retinoic acid-induced differentiation of F9 wild type and Ra-3-10 mutant teratocarcinoma cells", pages 1497-1504, see entire document.	1-38